UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS

TESIS DOCTORAL

Detección de especies potencialmente toxígenas de *Aspergillus* y *Fusarium* en maíz y avena. Diseño de nuevas estrategias sostenibles para su control

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

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Madrid

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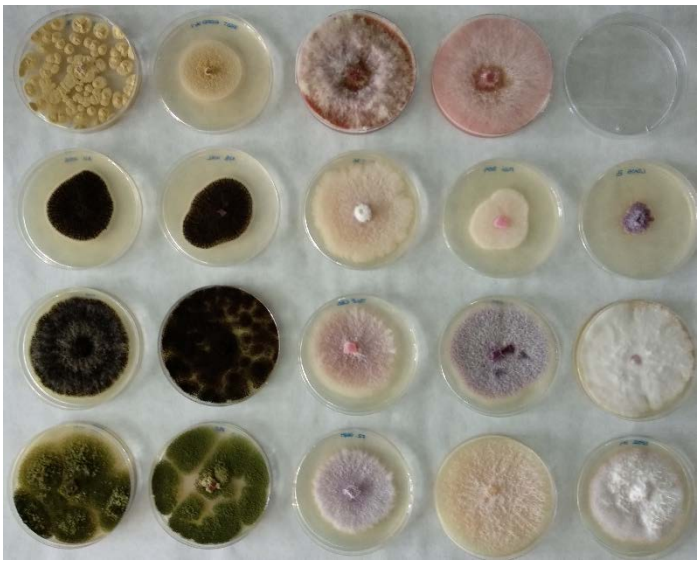

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Tesis doctoral presentada por Marta García Díaz para optar al grado de Doctora en Microbiología y Parasitología por la Universidad Complutense de Madrid

MADRID, 2020

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Universidad Complutense de Madrid

"Me parece haber sido sólo un niño jugando en la orilla del mar, divirtiéndose y buscando una piedra más lisa o una concha más bonita de lo normal, mientras el gran océano de la verdad yacía ante mis ojos con todo por descubrir"

Isaac Newton

"La agricultura es la profesión propia del sabio, la más adecuada al sencillo y la ocupación más digna para todo hombre libre"

Cicerón

A todas aquellas personas que me han acompañado en esta aventura, en especial a Sara, a Javi y a mis padres y hermanos.

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ÍNDICE

ABREVIATURAS

Además de las unidades y abreviaturas aceptadas por el Sistema Internacional de Unidades (SI), en esta Tesis se han utilizado las siguientes abreviaturas.

AE	Aceite Esencial
AF	Aflatoxinas
APPCC	Análisis de Peligros y Puntos de Control Crítico
a_w	Actividad de agua
BPA	Buenas Prácticas Agrícolas
CE	Comisión Europea
CYA	Agar Czapek Extracto de Levadura
DON	Deoxinivalenol
FAO	Organización de las Naciones Unidas para la Agricultura y la Alimentación
FB	Fumonisinas tipo B
FUM	Fumonisinas
GRAS	Generalmente Reconocido como Seguro
HL	Hidrolato
HPLC	Cromatografía Líquida de Alta Resolución
HPLC-FLD	Cromatografía Líquida de Alta Resolución con Detector Fluométrico
HT-2	Toxina HT-2
IARC	Agencia Internacional Investigación del Cáncer
LC-MS/MS	Cromatografía Líquida acoplada a Espectrometría de Masas
NIV	Nivalenol
NP	Nano Partículas
OMS	Organización Mundial de la Salud
OTA	Ocratoxina A
OV	*Origanum virens*
RASFF	Sistema de Alerta Rápida de Alimentos y Piensos
SM	*Satureja montana*
TCT	Tricotecenos
TEO	Aceite Esencial de Tomillo
TLC	Cromatografía en Capa Fina
T-2	Toxina T-2
UE	Unión Europea
ZEA	Zearalenona

ABBREVIATIONS

In this Thesis, in adition to the units and abbreviations accepted by the International System of Units (SI), the following abbreviations have been used.

AF	Aflatoxins
a_w	Water activity
CE	European Commission
CFU	Colony Forming Units
CYA	Czapek Yeast Agar
DLS	Dynamic Light Scattering
DON	Deoxynivalenol
EO	Essential Oil
FAO	Food and Agriculture Organization of the United Nations
FB	Type B Fumonisins
FF	Female Flower
FID	Flame Ionization Detector
FUM	Fumonisins
GAP	Good Agricultural Practices
GRAS	Generally Recognized as Safe
HL	Hydrolate
HPLC	High Performance Liquid Chromatography
HPLC-FLD	Liquid Chromatography Analysis with Fluorimetric Detection
HT-2	HT-2 toxin
IARC	International Agency Research on Cancer
LC-MS/MS	Liquid Chromatography coupled Mass Spectrometry
LOD	Limit of Detection
MF	Male Flower
NTA	Nanoparticle Tracking Analysis
OD	Optical Density
OTA	Ochratoxin A
OV	*Origanum virens*
P	Plot
PDA	Potato Dextrose Agar
PDI	Polydipersity Index
PEG	Polyethylene Glycol
PICS	Purdue Improved Crop Storage
PRE	Pre-harvest
RTD	Rate to Detection
SM	*Satureja montana*
TAE	Tris Acetate EDTA
TCT	Trichothecenes
tef-1α	Elongation factor 1α

TEO	Thyme Essential Oil
TFH	Thin Film Hydration
TLC	Thin Layer Chromatography
TTD	Time to Detección
T-2	T-2 toxin
YES	Yeast Extract Sucrose
ZEA	Zearalenone

RESUMEN/SUMMARY

RESUMEN

Los cultivos de cereal están frecuentemente contaminados por diversas especies de hongos filamentosos, siendo los géneros *Fusarium* y *Aspergillu*s los más importantes por albergar las principales especies productoras de micotoxinas. La presencia de dichas especies en los granos supone un grave problema debido a que los cereales son una fuente básica de la alimentación tanto humana como animal. Si las condiciones de almacenamiento no son las adecuadas, los hongos presentes en los cereales pueden producir micotoxinas, generando una amenaza para la seguridad alimentaria, ocasionando efectos adversos en la salud de los seres humanos y animales y graves pérdidas económicas para el sector agrícola.

Dado el riesgo que presentan las micotoxinas, la Unión Europea ha establecido unos contenidos máximos permitidos de las mismas en los cereales y sus productos derivados. A su vez el *Codex alimentarius*, creado por la FAO, recoge diferentes medidas de protección, que pueden ayudar a minimizar el riesgo de contaminación por micotoxinas en los granos de cereal como es la aplicación de buenas prácticas agrícolas (BPA).

Cuando las condiciones de humedad y temperatura no pueden ser controladas durante el almacenamiento de los cereales suelen emplearse fungicidas convencionales para inhibir el crecimiento fúngico y, de esta manera, evitar la aparición de micotoxinas en los granos. Sin embargo, su uso está en el punto de mira, ya que estos compuestos pueden ser perjudiciales tanto para la salud humana y animal como para el medio ambiente. Además, en la actualidad los consumidores demandan productos más naturales, por lo que es necesaria la búsqueda de nuevos métodos de control eficaces y sostenibles con el medio ambiente.

Los extractos de plantas aromáticas, como por ejemplo los aceites esenciales (AE), han demostrado tener fuertes propiedades antifúngicas y además se ha descrito que son capaces de interferir en la biosíntesis de las micotoxinas. Estas cualidades junto con su baja toxicidad y su aceptación por parte de los consumidores, los hacen buenos candidatos para su aplicación como fungicidas naturales para controlar hongos productores de micotoxinas.

España es uno de los principales países productores de cereal a nivel mundial y el maíz y la avena son dos de los más importantes. La mayor cantidad de la producción de maíz se destina habitualmente para la fabricación de piensos, mientras que la producción de avena ha aumentado considerablemente en los últimos años debido a la demanda por parte de los consumidores de productos más saludables para la dieta.

En este contexto, el primer objetivo de esta Tesis Doctoral fue evaluar el riesgo de contaminación por micotoxinas que presentan el maíz y la avena en España. Para ello, en primer lugar, se analizó la contaminación por micotoxinas y sus hongos productores a largo del ciclo de cultivo del maíz. Fueron evaluadas tres etapas (antesis, cosecha y almacenamiento) durante tres cosechas consecutivas (2016-2018) en

diferentes parcelas situadas al Sur de Madrid. Esta explotación agrícola aplica BPA tanto en campo como durante el almacenamiento y, además, cuenta con un secadero y almacén de grano propios. Para determinar la presencia de las principales especies productoras de micotoxinas en el maíz se utilizaron protocolos de PCR específicos descritos con anterioridad o desarrollados durante este trabajo, como es el caso de los protocolos para *F. graminearum*, *F. langsethiae*, *F. fujikuroi*, *F. poae*, *F. sporotrichioides* y *F. temperatum*. Los niveles de micotoxinas se determinaron mediante LC-MS/MS.

Las especies detectadas en alguna de las etapas durante el ciclo del cultivo del maíz fueron *F. verticillioides F. proliferatum*, *F. graminearum*, *A. flavus*, *A. parasiticus*, *A. carbonarius* y especies del agregado *A. niger* (*A. niger* y *A. welwitschiae*). *Fusarium verticillioides* y *F. proliferatum*, especies potencialmente productoras de fumonisinas tipo B (FB), y *A. flavus* potencialmente productora de aflatoxinas (AF) fueron las especies más predominantes en todas las etapas evaluadas, destacando la presencia de *A. flavus* desde las primeras etapas del cultivo, tanto en flores masculinas como en femeninas. De todas las toxinas analizadas, las FB fueron las únicas detectadas tanto en muestras tomadas en el momento de la cosecha como en almacén, aunque con niveles muy bajos siempre por debajo de los establecidos por la legislación Europea. A pesar de la alta incidencia de *A. flavus*, no se detectaron AF en ninguna de las etapas del cultivo.

Los resultados alcanzados en este trabajo ponen de manifiesto la importancia de las BPA en el ciclo del cultivo del maíz para prevenir la contaminación por micotoxinas. Se ha demostrado que a pesar de que la contaminación por importantes especies toxígenas ocurre desde inicio del cultivo, el control de la temperatura y la humedad durante el almacenamiento de los granos de maíz, son claves para impedir el desarrollo de estos hongos y la consecuente producción de micotoxinas.

Por otro lado, se evaluó el estado actual del maíz y la avena en España con respecto a su riesgo de contaminación por micotoxinas. Para ello se determinó la presencia de las principales especies de *Fusarium* y *Aspergillus* potencialmente productoras de micotoxinas en estos cereales, tanto en muestras de campo recolectadas en las principales zonas cerealistas españolas durante tres campañas (2016-2018), como en muestras disponibles comercialmente en España. La detección de estas especies en las muestras de cereal se evaluó mediante protocolos de PCR específicos para cada especie. Las principales especies detectadas fueron *A. flavus*, *A. niger*, *F. verticillioides* y *F. proliferatum* y, en general, el porcentaje de contaminación fue mayor en las muestras procedentes de maíz que las de avena, tanto en el caso de las muestras de campo como en las comerciales. La presencia de estas especies potencialmente toxígenas indicaría un posible riesgo de contaminación por AF, ocratoxina A y FB en los productos de maíz y avena cultivados y consumidos en España.

Como ya se ha mencionado anteriormente, la Unión Europea establece unos límites respecto al contenido máximo permitido de algunas micotoxinas en cereales y sus derivados. Los estudios amplios de incidencia de micotoxinas en alimentos como el realizado en esta Tesis pueden ser muy útiles para considerar si es pertinente modificar

los límites planteados en la normativa. Los resultados obtenidos en este estudio indican que el posible riesgo de contaminación por micotoxinas en maíz y avena en España no es tan elevado como para tener que plantear una nueva legislación en avena o modificar los niveles vigentes permitidos en maíz y sus derivados. Además, se ha confirmado la importancia de las técnicas moleculares, como los protocolos de PCR específicos utilizados en este trabajo, para la realización de estudios de incidencia de hongos productores de micotoxinas, así como para su detección temprana con el fin de predecir el riesgo de contaminación por micotoxinas en productos alimentarios. En el caso de detectar especies productoras, se deberían establecer medidas estrictas de control para evitar el desarrollo de los hongos e impedir que las micotoxinas entren en la cadena alimentaria.

Durante la realización de los trabajos anteriormente mencionados, se aislaron una gran variedad de cepas de *Fusarium* y *Aspergillus* de las muestras de cereal analizadas, con el fin de realizar un estudio para comprobar posibles relaciones filogenéticas. Las principales especies aisladas e identificadas coinciden con las detectadas mediante las PCR específicas realizadas en los granos de cereal, siendo *F. verticillioides, F. proliferatum A. flavus* y las especies del agregado *A. niger* (*A. tubingensis*, *A. niger* y *A. welwitschiae*) las mayoritarias. El estudio filogenético se realizó utilizando el método de Neighbor-Joining y secuencias parciales del gen que codifica el factor de elongación-1α, la β-tubulina y la calmodulina para los aislados del género *Fusarium,* de *Aspergillus* sección *Flavi* y del agregado *A. niger,* respectivamente. Los estudios filogenéticos revelaron que no existe variabilidad intraespecífica en ningún caso, y no se pudieron establecer relaciones entre las fuentes de aislamiento o la ubicación del cultivo.

Otro de los objetivos de esta Tesis Doctoral fue evaluar el efecto de extractos de planta sobre el crecimiento de *A. flavus* y su capacidad para producir AF, con el fin de desarrollar un sistema eficaz y sostenible para controlar la contaminación por AF durante el almacenamiento de los granos de maíz, determinando cuáles serían las condiciones más apropiadas para su aplicación.

En primer lugar, se evaluó el efecto *in vitro* de AE e hidrolatos (HL) de seis plantas aromáticas sobre el crecimiento y la producción de AF de *A. flavus*. Los extractos analizados fueron obtenidos de *Rosmarinus officinalis*, *Thymus vulgaris*, *Satureja montana* (SM), *Origanum virens* (OV), *O. majoricum* y *O. vulgare*. Para ello se suplementó medio de cultivo CYA con diferentes cantidades de dichos compuestos hasta alcanzar concentraciones finales de 10, 100, 500 y 1000 μg/mL, y se calculó la tasa de crecimiento y la fase de latencia de *A. flavus* en todas las condiciones. La producción de AF se detectó por HPLC tras 6 días de incubación. Los resultados revelaron que, en general, el efecto de los AE es muy superior al de los HL, destacando los AE de SM y OV que fueron capaces de reducir tanto el crecimiento como la producción de AF de *A. flavus* incluso a bajas dosis (100 μg/mL).

Una vez conocida la eficacia de los AE de SM y OV, se planteó estudiar cómo afectan las condiciones de humedad a su capacidad para reducir el crecimiento y la producción de AF en *A. flavus*. Para ello, se realizó un ensayo *in vitro* con diferentes concentraciones de AE (350, 700 y 1000 μg/mL) a tres niveles de actividad de agua (0,94, 0,96 y 0,98 a_w). El crecimiento del hongo se valoró mediante espectrofotometría utilizando el Bioscreen C y la concentración de AF se determinó mediante HPLC-FLD. Estos análisis se llevaron a cabo en la Universidad de Cranfield (Reino Unido) bajo la dirección del Dr. Ángel Medina. En cierta medida, los AE de SM y OV retrasaron el crecimiento de *A. flavus* a las tres a_w analizadas, reduciéndose la producción de AF en todos los casos. Sin embargo, el efecto fue mucho más significativo a altos niveles de a_w, cuando la producción de AF por parte del hongo es máxima. Estos resultados ponen de manifiesto la utilidad de la aplicación de los AE de SM y OV, tanto para controlar la contaminación por AF en matrices alimentarias con elevada a_w, como cuando las condiciones de humedad durante el almacenamiento no pueden ser controladas.

Los AE son extractos altamente volátiles, por lo que es preciso desarrollar un correcto procedimiento para su aplicación y así alargar su efecto en el tiempo sobre las matrices alimentarias. Por tanto, el último objetivo de esta Tesis se centró en optimizar el método de aplicación de los AE seleccionados sobre los granos de maíz para controlar la contaminación por AF durante el almacenamiento de los mismos. Para estandarizar el proceso, la puesta a punto del método de aplicación se realizó con un AE comercial de tomillo (TEO). En primer lugar, se evaluaron los procedimientos de aplicación más sencillos (contacto directo, volatilización y pulverización) sobre granos de maíz previamente inoculados con *A. flavus* y se valoró su efecto tras 9 y 28 días de incubación. El crecimiento del hongo se estimó mediante un recuento de viables mientras que la concentración de AF producida se analizó mediante TLC. Los resultados obtenidos indicaron que es necesario que el TEO entre en contacto con los granos para obtener una eficacia máxima, y que su efecto antifúngico se pierde con el tiempo, lo que hace necesario proteger el AE antes de su aplicación. Por este motivo, siguiendo el mismo protocolo anterior, se evaluó la efectividad del TEO encapsulado por distintos materiales (goma arábica, gelatina, alginato y niosomas). La encapsulación en niosomas fue el único método que permitió que el AE redujera tanto el crecimiento como la producción de AF de *A. flavus* respecto al control no tratado. Los niosomas son sistemas basados en lípidos que no presentan toxicidad, son biodegradables y fáciles de manipular, por lo que son buenos candidatos a la hora de ser aplicados a las matrices alimentarias. Posteriormente, se realizó un estudio a mayor escala simulando las condiciones reales de almacenamiento de los granos para comprobar si realmente los AE de SM y OV encapsulados en niosomas eran efectivos para reducir la contaminación por AF. Para ello se colocaron 100 g de maíz inoculado con *A. flavus* en bolsas tejidas de polipropileno y se añadieron, en su caso, 500 μg/g de los AE de SM u OV encapsulados en niosomas. Las bolsas se almacenaron durante 90 días en cajas de plástico en las que se monitorizó la temperatura y humedad con una sonda a lo largo del experimento. El crecimiento de *A. flavus* y la concentración de AF

en los granos de maíz se evaluaron a los 45, 60, 75 y 90 días mediante el recuento de viables y TLC, respectivamente. Los resultados obtenidos confirman que el nuevo método desarrollado sería eficaz para prevenir la presencia de AF en los granos de maíz almacenados cuando las condiciones ambientales no pueden ser controladas. Ambos AE encapsulados en niosomas redujeron tanto el crecimiento como la producción de AF durante el periodo de incubación, prolongando considerablemente la eficacia que se había observado con respecto al AE no encapsulado. Cabe destacar el efecto del AE de SM con una reducción del crecimiento del 79 % a los 60 días de almacenamiento, frente al 52 % del AE de OV. La aplicación de los AE encapsulados fue capaz de reducir la presencia de AF durante 75 y 90 días en el caso del AE de OV y SM, respectivamente, con respecto al control.

Por tanto, en esta Tesis se ha confirmado la importancia de las BPA para reducir el riesgo ocasionado por la presencia de micotoxinas en los granos de cereal durante el almacenamiento de los mismos. Además, se ha puesto de manifiesto la utilidad de las técnicas moleculares para estudiar el estado de los cultivos en relación a la presencia de especies potencialmente toxígenas y detectar tempranamente estos hongos, de manera que se puedan tomar estrictas medidas de control y evitar la producción de toxinas durante el almacenamiento de los granos. Por otro lado, se ha desarrollado un nuevo método de control sostenible con el medio ambiente e inocuo para la salud basado en AE encapsulados que puede ser utilizado para reducir el riesgo por micotoxinas en los cereales cuando las condiciones ambientales no pueden ser controladas.

SUMMARY

Cereal crops are often contaminated by diverse fungal species, and *Fusarium* and *Aspergillus* genera are the most important due to they include the main mycotoxin-producing species. The presence of these fungal species in the grains supposes a serious problem since cereals are a major food source for both human and animal diets. In the cases when storage conditions are not adequate, contaminating fungi may produce mycotoxins. These compounds threaten food safety, as well as human and animal health, and cause important economic losses in agricultural sector.

Taking into account the risk posed by mycotoxins, the European Union has set the maximum limits in cereals and their derivatives. Moreover, the Codex Alimentarius, which was created by FAO, explains different measures, that can help to minimize mycotoxin contamination risk in cereal grains including the establishment of Good Agricultural Practices (GAP).

Whether humidity and temperature conditions during cereal storage cannot be controlled, conventional fungicides are often used to prevent fungal growth and, therefore, mycotoxin occurrence in the grains. However, fungicides are in the spotlight since they could be harmful compounds for human and animal health as well as for the environment. Currently, the consumers demand more natural products, which makes necessary the search for new effective and environmentally friendly control methods.

Aromatic plant extracts such as essential oils (EO), have been demonstrated to present not only antifungal properties but also to interfere in mycotoxin biosynthesis. This fact together with their low toxicity and their acceptance by consumers, make essential oils good candidates to be used as natural fungicides towards mycotoxigenic fungi.

Spain is one of the main cereal producers worldwide and maize and oat are two of the most important ones. Most of maize production is usually intended for animal feed fabrication, whereas oat production has considerably risen in the last few years due to consumers' demand for healthy products.

In this context, the first objective of this Thesis was to evaluate the risk posed by mycotoxin contamination in maize and oat in Spain. Initially, the occurrence of the main mycotoxins and their producing fungi was evaluated during the complete maize production cycle. Three production steps (anthesis, harvest and storage) were evaluated during three consecutive seasons (2016-2018) in different plots located in the South region of the Community of Madrid. This farm applies GAP both in the field and during storage and, it also presents its own dryer and grain store. In order to determine the occurrence of the main mycotoxin producing species several species specific PCR protocols were applied including those used to detect *F. graminearum*, *F. langsethiae*, *F. fujikuroi*, *F. poae*, *F. sporotrichioides* and *F. temperatum* which were optimized in this work. Mycotoxin levels were studied by LC-MS/MS.

Fusarium verticillioides F. proliferatum, F. graminearum, A. flavus, *A. parasiticus*, *A. carbonarius* and *A. niger* aggregate species (*A. niger* and *A. welwitschiae*) were detected in at least one step during maize production cycle. *Fusarium verticillioides* and *F. proliferatum*, which are potential producers of fumonisins (FB), and *A. flavus*, which is potential producer of aflatoxins (AF), were the most frequently detected species in all the stages of maize cycle. It is important to highlight that *A. flavus* was present since anthesis and was detected in both male and female flowers. Out of all the mycotoxins analyzed, only FB were detected both in harvest and postharvest samples, although the levels were very low and always under the limits established by European legislation. Despite the high incidence of *A. flavus*, AF were not detected in any of the steps of maize production cycle.

The results obtained in this work revealed the importance of GAP in maize production cycle to avoid mycotoxin contamination. Despite contamination by relevant mycotoxigenic fungi occur in the first steps of maize cultivation process, the control of temperature and humidity levels during storage of maize grains is crucial to prevent fungal development and the consequent mycotoxin production.

Furthermore, the state of maize and oat crops in Spain regarding their risk of contamination by mycotoxins was evaluated. The presence of potential mycotoxigenic *Fusarium* and *Aspergillus* species was determined in these cereals, both in field samples collected in the main Spanish cereal regions during three seasons (2016-2018), and in samples commercially available in the Spanish market. Species specific PCR protocols were applied to detect these species in cereal samples. The most frequently detected species were *A. flavus*, *A. niger*, *F. verticillioides* and *F. proliferatum*. In general, maize presents higher percentages of contamination than oat, both in field and commercial samples. The presence of these species might be an indicator of contamination of maize and oat products from Spain by AF, ochratoxin A, and FB.

As mentioned above, the European Commission set the maximum allowed limits of some mycotoxins in cereals and their derivatives. Wide studies on the occurrence of mycotoxins in food products such as the presented in this Thesis are very useful to consider if it is appropriate to modify the limits established by the legislation. The results obtained indicate that the risk posed by mycotoxin contamination of maize and oat in Spain is not high enough neither to plan a new legislation regarding oat nor to modify the current levels allowed in maize and its by-products. Moreover, the application of molecular methods (such as the species specific PCR protocols used in this work) has been revealed as a useful tool in surveys to determine species occurrence, and for their early detection in order to predict the risk of mycotoxin contamination in food products. In the case mycotoxin producing species were detected, strict control measures might be applied to avoid fungal development to avoid mycotoxins entering the food chain.

During the development of the aforementioned assays, a wide variety of *Aspergillus* and *Fusarium* isolates were obtained which were used to perform a

phylogenetic study to unravel possible relationships among them. The main isolated and identified species are in agreement with those detected using species specific PCR protocols applied in cereal grains, being *F. verticillioides*, *F. proliferatum A. flavus* and *A. niger aggregate species* (*A. tubingensis*, *A. niger* y *A. welwitschiae*) the most common. The Neighbour-Joining method was used to construct a phylogenetic tree based on the partial sequences of the elongation factor-1α, tubulin and calmodulin encoding genes in the case of *Fusarium*, *Aspergillus* section *Flavi*, and *A. niger* aggregate species, respectively. Phylogenetic studies revealed no intraspecific variability in any case and it was not possible to establish relationships among the isolates regarding neither isolation source nor crop location.

Other objective planned in this Thesis was to evaluate the effect of plant extracts on *A. flavus* growth and its ability to produce AF, in order to develop an efficient and sustainable method to prevent AF contamination of maize grains during storage and to determine the most appropriate conditions for their application.

First of all, the in vitro effect of the EO and hydrolates (HL) from six aromatic plants on growth and AF production by *A. flavus*. The extracts analysed were obtained from *Rosmarinus officinalis*, *Thymus vulgaris*, *Satureja montana* (SM), *Origanum virens* (OV), *O. majoricum* y *O. vulgare.* To this end, CYA medium was supplemented with different amounts of these extracts at final concentrations of 10, 100, 500, and 1000 μg/mL and *A. flavus* growth rate and lag phase was calculated at all conditions tested. AF concentration was determined by HPLC after 6 days of incubation. The results obtained revealed that EO were far more effective than HL. SM and OV EO stood out since they were able to reduce both growth and AF production by *A. flavus* even at low doses (100 μg/mL).

Once the efficiency of SM and OV EO was demonstrated, the effect of humidity conditions on their ability to reduce *A. flavus* growth and AF production was subsequently evaluated. An in vitro assay was performed testing different EO concentrations (350, 700 y 1000 μg/mL) at three water activity levels (0.94, 0.96 y 0.98 a_w). Fungal growth was assessed by spectrophotometry using a Bioscreen C and AF concentration was determined by HPLC-FLD. These analyses were performed in Cranfield University (UK) under the supervision of Dr. Ángel Medina. Both SM and OV EO retarded, to some extent, fungal growth at the three a_w levels tested, and AF concentration was reduced in all cases. However, the effect was more significant at high levels of a_w, in which AF production by *A. flavus* reached its maximum. These results indicated that the application of SM and OV EO might be useful to control AF contamination in food matrices with high a_w or in the case humidity conditions during storage could not be controlled.

EO are highly volatile extracts and, therefore, it is essential to develop a correct application procedure to extend their effect when they are applied over food matrices. Consequently, the last objective of this Thesis was focused on the optimization of the most suitable application method on maize grains for the selected EO in order to

control AF contamination during storage. The standardization of the process was performed using a commercial thyme essential oil (TEO). First of all, the most simple application procedures were studied (direct contact, volatilization, and pulverization) over maize grains previously inoculated by *A. flavus* and their effect was evaluated after 9 and 28 days of incubation. Fungal growth was estimated by viable counts whereas toxin concentration was analyzed by TLC. The results showed that the contact between TEO and the grains is necessary to reach the highest efficiency and the antifungal effect is lost over time, which makes necessary the protection of the EO before its application. For this reason, the next step was to evaluate the efficiency of TEO encapsulated by different materials (Arabic gum, gelatin, alginate, and niosomes) following the same protocol mentioned before. Niosome encapsulation was the only method that allowed the TEO to reduce both *A. flavus* growth and AF production with respect to untreated controls. Niosomes are lipid-based systems with no toxicity, biodegradables, and easy to manipulate, and, therefore, good candidates to be applied over food matrices. Subsequently, the efficiency of niosome-encapsulated SM and OV EO to prevent AF contamination was tested at a major scale simulating the real storage conditions of maize grains. For that purpose, polypropylene bags were filled with 100 g of inoculated maize and, when necessary, 500 μg/g of niosome encapsulated SM or OV EOs were included. The bags were incubated in plastic boxes during 90 days and temperature and humidity were monitored across the experimental time. Fungal growth and AF accumulation in maize grains were tested by viable counts and TLC, respectively, after 45, 60, 75 and 90 days of incubation. The results obtained confirmed that the new developed method would be effective to prevent AF contamination of maize grains when storage environmental conditions could not be controlled. Both encapsulated EO were able to reduce fungal growth and AF production during storage time, extending the efficiency previously reported in vitro in the case of non-encapsulated EO. It is important to highlight the effect of SM EO after 60 days of incubation with a reduction in fungal growth of 79 %, whereas this reduction was 52 % with OV EO. The application of encapsulated EO were able to reduce AF presence after 75 and 90 % in the case of OV and SM EO, respectively, when compared to untreated control.

In conclusion, in this Thesis, the relevance of GAP to avoid mycotoxin contamination in grain cereals during storage was confirmed. Moreover, it has been demonstrated the usefulness of molecular techniques to study the state of the crops in relation to the presence of potentially toxigenic species and to detect these fungi, in order to establish strict control measures to prevent toxin production during grain storage. Finally, a new sustainable and safe control method based on encapsulated EO, which could be used to reduce mycotoxin risk in cereals when environmental conditions cannot be controlled.

ESTRUCTURA DE LA TESIS

La presente Tesis Doctoral está estructurada de la siguiente forma: Los principales resultados están recogidos en cuatro capítulos, que corresponden a tres artículos científicos ya publicados en revistas indexadas y a uno en preparación. Los cuatro capítulos están relacionados a través de la introducción y discusión general. Los capítulos han sido maquetados para mantener la misma estructura a lo largo de toda la Tesis, pero el contenido de los trabajos publicados se ha mantenido integro. El capítulo 3 consta de material suplementario, el cual se ha incluido al final del capítulo. El listado de capítulos contenidos en la Tesis se describe a continuación:

Capítulo 1: Marta García-Díaz, Jéssica Gil-Serna, Covadonga Vázquez, María Nieves Botia, Belén Patiño. A comprehensive study on the occurrence of mycotoxins and their producing fungi during the maize production cycle in Spain. Microorganisms 2020, 8, 141; doi: 10.3390/microorganisms8010141.

Capítulo 2: Marta García-Díaz, Jéssica Gil-Serna, Covadonga Vázquez, Belén Patiño. Occurrence of *Fusarium* and *Aspergillus* species in maize and oat samples and their subproducts in Spain. (En preparación).

Capítulo 3: Marta García-Díaz, Belén Patiño, Covadonga Vázquez, Jéssica Gil-Serna. A novel niosome-encapsulated essential oil formulation to prevent *Aspergillus flavus* growth and aflatoxin contamination of maize grains during storage. 2019. Toxins 2019, 11, 646; doi: 10.3390/toxins11110646.

Capítulo 4: Marta García-Díaz, Jéssica Gil-Serna, Belén Patiño, Esther García-Cela, Naresh Magan, Ángel Medina. Assessment of the effect of *Satureja montana* and *Origanum virens* essential oils on *Aspergillus flavus* growth and aflatoxin production at different water activities. 2020. Toxins 2020, 12, 142; doi: 10.3390/toxins12030142.

INTRODUCCIÓN GENERAL

1. Micotoxinas

Las micotoxinas son metabolitos secundarios producidos por hongos filamentosos cuya ingestión, inhalación o absorción cutánea reduce la actividad, hace enfermar o causa la muerte de seres humanos y animales [1]. Los efectos producidos por las micotoxinas se denominan micotoxicosis y pueden ser crónicas o agudas según el nivel y tiempo de exposición a las mismas, pudiendo afectar al sistema nervioso central, sistema cardiovascular, pulmonar y tracto digestivo, entre otros. Además algunas toxinas actúan como agentes cancerígenos, mutagénicos, teratogénicos e inmunosupresores [2]. Debido a su toxicidad, la presencia de micotoxinas en las materias primas, los alimentos y los piensos destinados a alimentación animal, representa un grave riesgo para la seguridad alimentaria, además de ocasionar grandes pérdidas económicas en los sectores agrícolas y ganaderos de todo el mundo [3,4].

Los hongos toxígenos se desarrollan en diversos tipos de cultivos, cuando se dan condiciones de humedad y temperatura adecuadas [5]. Debido a esto, la contaminación por micotoxinas está muy extendida en los cereales, los frutos secos, las semillas de oleaginosas, algunas frutas, el café, el cacao y en las especias, donde el crecimiento fúngico y la producción de micotoxinas puede ocurrir a lo largo de toda la cadena alimentaria, desde los cultivos en el campo hasta el almacenamiento [6]. Además la exposición humana a las micotoxinas puede deberse no sólo al consumo de alimentos vegetales contaminados con toxinas, sino también al consumo de productos de origen animal contaminados, debido a la transferencia de micotoxinas y sus derivados a los animales por el consumo de piensos contaminados [7]. El metabolismo de las micotoxinas ingeridas a través de los piensos podría dar lugar a su acumulación en diferentes órganos o tejidos animales, entrando en la cadena alimentaria a través de la carne, la leche o los huevos [8].

Los principales géneros fúngicos productores de micotoxinas son *Aspergillus*, *Fusarium*, *Penicillium* y *Alternaria* [8], habiéndose identificado hasta la fecha aproximadamente 10.000 especies micotoxígenas capaces de producir más de 500 micotoxinas. Se estima además, que podría haber otras 1.000 especies potencialmente productoras [9]. Actualmente, las micotoxinas que causan mayor preocupación a nivel global son las aflatoxinas (AF), las fumonisinas tipo B (FB), los tricotecenos (TCT), la ocratoxina A (OTA), la zearalenona (ZEA) y la patulina [10,11].

1.1. Principales micotoxinas detectadas en cereales

Las micotoxinas que aparecen con mayor frecuencia en los granos de cereal son las AF, FB, TCT, OTA y ZEA [4,5]. Es importante destacar que una determinada micotoxina puede ser producida por más de una especie toxígena y que una misma especie de hongo es capaz de producir más de un tipo de micotoxina [12], por lo que la coexistencia de varias micotoxinas en un mismo alimento es algo habitual.

Los hongos toxígenos que contaminan los cultivos de cereal pueden ser patógenos, como por ejemplo las especies del género *Fusarium*, o saprófitos como las especies de *Aspergillus* y *Penicillium* [13]. Las especies de *Fusarium* son también conocidas como hongos pre-cosecha [14], ya que es durante el ciclo de cultivo cuando se produce la colonización y el desarrollo de *Fusarium*. Esto es debido a que como especies fitopatógenas que son, van a participan en la captura de los recursos primarios y, por lo tanto, su establecimiento en la planta es fundamental para la infección [15]. Por el contrario las especies del género *Aspergillus* secciones *Flavi, Circumdati* y *Nigri,* han sido descritas tradicionalmente como post-cosecha o de almacenamiento, ya que son especies xerófilas capaces de colonizar nichos donde hay menos competencia. Los granos de cereal dañados por plagas o huéspedes estresados por la sequía son el nicho propicio para la colonización de estas especies [16].

Entre las micotoxinas más importantes producidas por las especies de *Aspergillus* en cereales se encuentran las AF y la OTA, mientras que las micotoxinas más importantes producidas por las especies de *Fusarium* son los TCT y las FB, y en menor medida la ZEA [4,5].

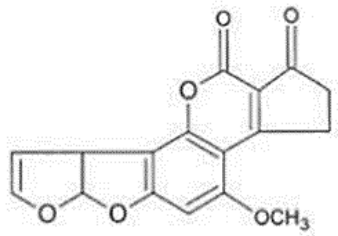
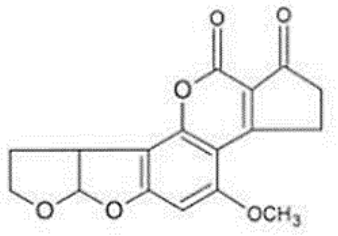
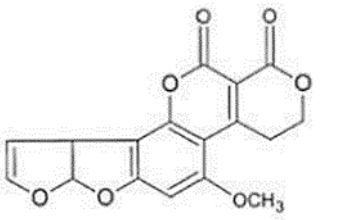
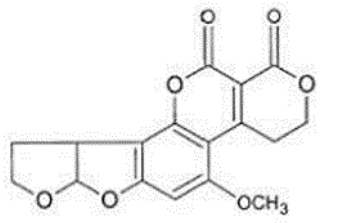
- **Aflatoxinas**

La contaminación de los alimentos y piensos con AF ha sido ampliamente estudiada desde la década de los 60, cuando se produjo la muerte de más de 100.000 pavos jóvenes en diferentes granjas avícolas de Inglaterra debido al consumo de harina de cacahuete contaminada con AF [17].

Hay más de 20 tipos de AF conocidas distribuidas por todo el mundo, pero las más importantes son las AFB_1, B_2, G_1 y G_2 [18]. Las AF tipo B y G se caracterizan por emitir fluorescencia bajo la luz UV, siendo en las tipo B azul (B: *Blue*), mientras que en las tipo G es verde (G: *Green*).

Las AF son compuestos orgánicos heterocíclicos de bajo peso molecular. Su esqueleto básico es un anillo de furano unido al núcleo de una cumarina. Las AF de la sección B tienen un anillo de ciclopentona en la molécula, mientras que las de la serie G tienen un anillo de lactona [2,19]. Además las AFB_1 y G_1, tienen un doble enlace del que carecen las B_2 y G_2. Sus propiedades físicos-químicas, así como su estructura molecular se detallan en la Tabla 1.

Tabla 1. Estructura química de las aflatoxinas, formula química, masa atómica, densidad y punto de fusión.

	**Aflatoxina B_1**	
	Fórmula molecular	**$C_{17}H_{12}O_6$**
	Masa atómica	**312,06 Da**
	Densidad	**1,6 ± 0,1 g/cm³**
	Punto de fusión	**528,2 ± 50,0 °C (760 mm Hg)**
	**Aflatoxina B_2**	
	Fórmula molecular	**$C_{17}H_{14}O_6$**
	Masa atómica	**314,08 Da**
	Densidad	**1,5 ± 0,1 g/cm³**
	Punto de fusión	**521,0 ± 50,0 °C (760 mm Hg)**
	**Aflatoxina G_1**	
	Fórmula molecular	**$C_{17}H_{12}O_7$**
	Masa atómica	**328,06 Da**
	Densidad	**1,6 ± 0,1 g/cm³**
	Punto de fusión	**612,1 ± 55,0 °C (760 mm Hg)**
	**Aflatoxina G_2**	
	Fórmula molecular	**$C_{17}H_{14}O_7$**
	Masa atómica	**330,07 Da**
	Densidad	**1,6 ± 0,1 g/cm³**
	Punto de fusión	**602,5 ± 50,0 °C (760 mm Hg)**

Datos obtenidos de la base de datos Chemspider [20].

Estas toxinas pueden acceder al organismo por ingestión, inhalación o contacto con la piel, dando lugar a una sobre-activación de la respuesta inflamatoria [18]. La contaminación por AF supone una grave amenaza para la salud humana y animal, pudiendo causar varios tipos de cáncer. De entre ellas, destaca la AFB_1 que ha sido identificada por la Agencia Internacional para la Investigación del Cáncer (IARC por sus siglas en inglés) como el carcinógeno natural más potente conocido para el ser humano [21].

Las aflatoxicosis en animales son debidas al consumo de piensos contaminados con AF, y la gravedad de la enfermedad depende de la especie, la edad, el sexo y el estado nutricional del animal. El órgano más afectado es el hígado aunque también se han observado síntomas de disfunción gastrointestinal, reducción de la reproductividad, anemia, así como una disminución en la producción de leche y huevos [18,22]. Las aflatoxicosis en humanos han sido ampliamente estudiadas y se ha descrito que una exposición de baja a moderada causa problemas de digestión y retraso en el crecimiento. Sin embargo, la ingestión de dosis más elevadas de AF puede dar lugar a una aflatoxicosis aguda que, en los casos más graves, puede desembocar en una insuficiencia hepática fulminante. La aflatoxicosis debida a una exposición crónica a estas micotoxinas genera malformaciones congénitas y cáncer [18,22].

Las AF son producidas por varias especies de *Aspergillus* de la sección *Flavi*; *A. flavus* y *A. parasiticus* son las principales especies productoras, destacando *A. flavus* por ser una especie contaminante muy común en los productos agrícolas. *Aspergillus bombycis*, *A. pseudotamarii, A. nomius, A. ochraceoroseus, A. arachidicola, A. minisclerotigenes A. parvisclerotigenus* y *A. pseudocaelatus*, han sido descritas más recientemente como especies productoras de AF, pero hasta el momento se han encontrado con menor frecuencia contaminando productos alimentarios [2,23-25].

El cereal más contaminado con las especies productoras de aflatoxinas es el maíz [26].

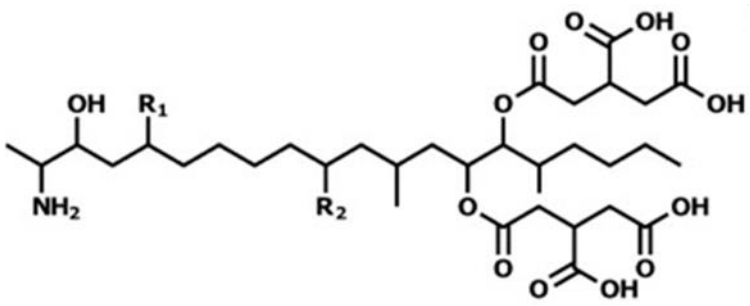
- **<u>Fumonisinas</u>**

Las fumonisinas fueron descubiertas en 1988 en la antigua región de Transkei (Sudáfrica), al estudiar la relación entre el cáncer de esófago en los seres humanos, y el consumo de maíz contaminado con *F. verticillioides* [27].

Existen más de 28 tipos de fumonisinas, que se clasifican químicamente en cuatro series: A, B, C y P, siendo la serie B la más abundante en los cereales. Dentro de esta se distinguen 6 tipos (B_1, B_2, B_3, B_4, B_5 y B_6). La fumonisinas tipo B_1 (FB_1), B_2 (FB_2) y B_3 (FB_3) se encuentran de forma habitual en los cultivos, siendo la FB_1 la más importante. Esta micotoxina constituye un 70 % del contenido de todas las FB en los alimentos y es la más tóxica. Por el contrario, los niveles de FB_2 y FB_3 que se encuentran comúnmente en los alimentos suelen ser bajos [28].

Las fumonisinas de tipo B están constituidas por un esqueleto lineal de 20 átomos de carbono, con un grupo amina en el carbono C_2, junto con dos ácidos tricarboxílicos esterificados en los carbonos C_{14} y C_{15}. Estas moléculas difieren unas de otras en la presencia o ausencia de un grupo hidroxilo en los carbonos C_5 y C_{10} [29,30]. La estructura molecular y las propiedades físicos-químicas de las principales FB se detallan en la Tabla 2.

Tabla 2. Estructura química de las fumonisinas, formula química, masa atómica, densidad y punto de fusión



	Fumonisina B_1	**Fumonisina B_2**	**Fumonisina B_3**
R1	**OH**	**OH**	**H**
R2	**OH**	**H**	**OH**
Fórmula molecular	**$C_{34}H_{59}NO_{15}$**	**$C_{34}H_{59}NO_{14}$**	**$C_{34}H_{59}NO_{14}$**
Masa atómica	**721,39 Da**	**705,39 Da**	**705,83 Da**
Densidad	**1,3 ± 0,1 g/cm³**	**1,2 ± 0,1 g/cm³**	**1,2 ± 0,1 g/cm³**
Punto de fusión (760 mm Hg)	**924,9 ± 65,0 °C**	**864,4 ± 65,0 °C**	**867,0 ± 65,0 °C**

Datos obtenidos de la base de datos Chemspider [20]

Las FB están clasificadas como carcinógenos del grupo 2B (posibles carcinógenos humanos) por la IARC [21]. Afectan tanto a humanos como al ganado y causan múltiples enfermedades agudas y crónicas [31]. El mecanismo de la acción tóxica de las FB se atribuye a la interferencia con el metabolismo de la esfingosina y la esfinganina, lo que perturba el metabolismo de los esfingolípidos. La estructura química de las FB es similar a la de los esfingolípidos de membrana, y su presencia puede bloquear la ruta de síntesis de los mismos [31,32].

Se han atribuido varias patologías debidas a la exposición a las FB, principalmente en el ganado, como la leucoencefalomalacia en caballos, edema pulmonar en cerdos, y efectos hepatotóxicos y cáncer de hígado en ratas. Además de estos efectos agudos, una exposición crónica a las FB se asocia con el retraso en el crecimiento y bajada de peso en cerdos [21]. En los seres humanos, no hay evidencias científicas claras de las consecuencias que provocan las FB en la salud, aunque algunos estudios han demostrado una asociación entre el consumo de maíz altamente contaminado con FB y diversas enfermedades. Ejemplos de esto son la alta incidencia de cáncer de esófago en Sudáfrica, China e Irán, o un aumento de defectos congénitos en el tubo neural en los partos de mujeres en Guatemala, China y Sudáfrica [31].

Las FB son producidas principalmente por diversas especies del género *Fusarium* dentro del complejo de especies *Fusarium fujikuroi* [33]. Las especies productoras de FB más importantes son *F. verticillioides* y *F. proliferatum* [34]. Estas especies fitopatógenas son las responsables de diversas enfermedades en los cultivos agrícolas, destacando su presencia en los cultivos de cereal de todo el mundo, principalmente en las zonas productoras de maíz [35]. Otras especies de *Fusarium* productoras de FB son *F. fujikuroi, F. globosum, F. napiforme, F. nygamai, F. subglutinans* y *F. temperatum* [28,36,37]. Algunas especies de *Aspergillus* de la sección *Nigri* como *A. welwitschiae* y *A. niger* también pueden producir fumonisinas tipo B_2 [38,39].

- **Tricotecenos**

El descubrimiento de los TCT se produjo con la descripción de la enfermedad denominada Aleucia Alimentaria Toxica, que causo numerosas muertes en la década de 1940 en Rusia [40]. Los TCT son un grupo complejo de sesquiterpenoides químicamente relacionados que se dividen en tipos A y B. Los tipo A tienen una sustitución de hidroxilo o éster o ningún oxígeno en el carbono C_8, mientras que los tipo B tienen un grupo ceto (carbonilo) en el carbono C_8. Los TCT tipo B se producen generalmente en cantidades más elevadas, pero son menos tóxicos que los compuestos de tipo A. Dentro de los TCT tipo A se encuentra principalmente el diacetoxiscirpenol y las toxinas T-2 y HT-2, y dentro de los de tipo B el deoxinivalenol (DON) y el nivalenol (NIV) [32].

La presencia de TCT en los cereales se ha asociado tanto a problemas sanitarios en el ganado como a micotoxicosis humanas. Los síntomas descritos en los animales incluyen retraso en el crecimiento, trastornos reproductivos, supresión del sistema inmunológico, vómitos, hemorragias, diarrea e incluso la muerte. Por otro lado, la exposición a TCT en la dieta en humanos se relaciona con náuseas, diarrea, dolor abdominal y fiebre [32].

Los TCT son producidos por diversas especies del genero *Fusarium*. Los TCT tipo A son sintetizados principalmente por *F. acuminatum, F. langsethiae, F. poae*, *F. sambucinum, F. sporotrichioide*s, *F. venenatum* y *F. equiseti,* mientras que los tipo B son producidos por *F. graminearum F. culmorum, F. crookwellense* y *F. pseudograminearum* [32].

La incidencia de DON en cereales se da principalmente en los cultivos de maíz, trigo y cebada [41], mientras que las toxinas T-2 y HT-2 aparecen con mucha frecuencia en los cultivos de avena del Norte de Europa [42].

- **Ocratoxina A**

La ocratoxina A fue descubierta en 1965 por Van der Merwe y colaboradores [43]. Esta toxina es el agente causal de la enfermedad renal conocida como Nefropatía Endémica de los Balcanes [44]. La OTA es una molécula formada por una dihidroisocumarina unida mediante un enlace amida a una molécula de L–β–fenilalanina. Esta micotoxina es una molécula muy estable que puede resistir altas temperaturas, lo que hace muy difícil su eliminación en los tratamientos térmicos habituales llevados a cabo en la industria alimentaria [45].

La ingestión de OTA a través de los alimentos y los piensos causa toxicidad renal, nefropatías, hepatotoxicidad, teratogenicidad e inmunotoxicidad, y además está clasificada como un probable carcinógeno en humanos dentro del grupo 2B [21].

La OTA es producida por diferentes especies de los géneros *Aspergillus* y *Penicillium* aunque las condiciones óptimas de producción de ambos géneros son muy diferentes. En climas cálidos son más relevantes las especies del género *Aspergillus* siendo en las secciones *Circumdati* y *Nigri* donde se encuentran las principales especies productoras, *A. westerdijkiae, A. steynii, A. carbonarius, A. niger* y *A. welwitschiae* [44,46,47]. En el caso de *Penicillium* las principales especies productoras de OTA son *P. nordicum* y *P. verrucosum* [48].

La presencia de OTA se ha descrito en casi todos los cereales como por ejemplo la cebada, el trigo, el maíz, el arroz, el centeno y la avena [49,50].

- **Zearalenona**

La zearalenona fue descubierta en asociación con un brote de síndrome estrogénico en los cerdos, y fue denominada en un primer momento como "sustancia estrogénica F-2". En 1966 fue aislada y caracterizada por primera vez a partir de maíz contaminado por *F. graminearum*. La ZEA es una lactona de ácido resorcílico cuya fórmula es 6-[10-hidroxi-6-oxo-trans-1-undecenil]-β-resorcílico [32].

Esta micotoxina estrogénica tiene baja toxicidad aguda y no causa micotoxicosis mortales. Sin embargo, la ZEA es una de las micotoxinas más importante desde el punto de vista económico debido a su asociación con las anomalías reproductivas, principalmente en los cerdos. La ingesta de cereales contaminados con ZEA puede ocasionar problemas hormonales en ganado porcino, ovino y bovino, tales como el agrandamiento de las glándulas mamarias y los genitales, la atrofia de los ovarios o los testículos, la infertilidad, la reducción del tamaño de la camada y la reducción del peso de la descendencia [32]. En estudios con animales de experimentación se han obtenido pocas pruebas de la carcinogenicidad de la ZEA [51].

La ZEA es producida por diversas especies dentro del complejo *F. graminearum*, muchas de las cuales también producen TCT tipo B. Por lo tanto, la ZEA se encuentra frecuentemente en los cereales junto con DON o NIV. Las principales especies productoras de ZEA son *F. crookwellense, F. culmorum, F. equiseti, F. graminearum, F. pseudograminearum* y *F. semitectum* [32].

Dentro de los cereales se considera que el maíz y los productos a base de maíz son la principal fuente de exposición a la ZEA, aunque esta toxina también puede aparecer en otros cereales como la cebada, el trigo, la avena, el arroz y el sorgo en climas cálidos y templados [52].

1.2. Factores que afectan al desarrollo fúngico y a la producción de micotoxinas durante el cultivo y en el almacenamiento de los cereales

El crecimiento fúngico y la producción de micotoxinas dependen principalmente de tres tipos de factores que se clasifican en biológicos, físicos y químicos.

- **Factores biológicos**. La susceptibilidad del grano de cereal a ser colonizado y la competencia que se establece entre las diferentes especies colonizadoras por los nutrientes del medio, son determinantes para que los hongos puedan crecer y producir toxinas [53]. Otro factor biológico muy importante, tanto en campo como en almacén, es la presencia de insectos. Los insectos actúan como vector de entrada produciendo daños en las plantas y granos de cereal al alimentarse de los mismos, facilitando la entrada de hongos toxígenos a través de las heridas causadas [54]. En estos últimos años se ha demostrado que *Ostrinia nubilalis* (taladro del maíz) y *Helicoverta armígera* (gusano cogollero) juegan un papel muy importante en la diseminación de esporas de *F. verticillioides* y *A. flavus* en las plantaciones de maíz cultivadas en zonas de clima templado [55]. Además, algunas investigaciones muestran que estas larvas se pueden alimentar de micelios de *F. verticillioides* contribuyendo a la diseminación de las esporas por sus excrementos [56]. Dentro de los factores biológicos también se incluyen los restos de cosecha, que van actuar como reservorio de las especies productoras de micotoxinas. Ya se ha mencionado anteriormente que *Aspergillus* y *Fusarium* son las especies micotoxígenas más frecuentes en los cereales y, ambos géneros, pueden crecer saprófitamente en los restos de la cosecha. Estos residuos sirven de depósito para los hongos, permitiéndole pasar el invierno en forma de esclerocios (*Aspergillus*) y clamidosporas (*Fusarium*). Cuando las condiciones son favorables las esporas germinan y pueden volver a invadir las plantas de cereal al ser transmitidas por el aire, los insectos o los aperos de labranza [15,36,57].

- **Factores físicos**. Los principales factores ecofisiológicos que afectan al crecimiento de los hongos y a la producción de micotoxinas son la humedad y la temperatura [57-59]. Estos factores determinan tanto la germinación de las esporas como la tasa de crecimiento de los hongos y pueden afectar directamente a la biosíntesis de las toxinas. Los valores umbral de actividad de agua (a_w) y temperatura que determinan el crecimiento de los hongos y la producción de toxinas difieren entre las diferentes especies fúngicas y, a su vez, el óptimo de crecimiento puede ser diferente al óptimo de producción de toxinas, siendo el rango óptimo de producción de toxinas más estrecho que el del crecimiento fúngico [60,61]. Por lo tanto, es difícil describir un único conjunto de condiciones óptimas para el crecimiento de hongos y producción de micotoxinas [62]. La capacidad de los hongos para crecer y sintetizar micotoxinas en los cultivos de cereal depende de las condiciones ambientales, del área geográfica y, además, difiere significativamente entre estaciones secas y húmedas [5]. La zona mediterránea se caracteriza por una elevada humedad y temperaturas moderadas, siendo éstas las condiciones idóneas para el crecimiento de algunos hongos y la aparición de micotoxinas [63]. Además la desertificación y las fluctuaciones de los ciclos húmedos y secos asociadas al cambio climático, tienen un fuerte impacto en el ciclo de vida de todos los microorganismos, incluidos los hongos toxígenos [64]. En Europa, la presencia de AF en cereales ha adquirido una importancia creciente en estos últimos años como consecuencia del aumento de las temperaturas medias, y se prevé que en los próximos 30 años aumente considerablemente el riesgo de

contaminación por AF en el maíz de Europa meridional y central. Así mismo, el perfil de las especies micotoxígenas de *Fusarium* en el trigo está en continuo cambio, con una creciente y preocupante contaminación por *F. graminearum* en Europa central y septentrional [65]. Son varios los estudios que han demostrado que el patrón de incidencia de especies toxígenas debido al cambio climático no solo está relacionado con variaciones en la temperatura y la humedad relativa, sino que los niveles de CO_2 también tienen un papel determinante [13,65,66]. Se ha visto que el aumento del CO_2 atmosférico en el marco del cambio, puede influir significativamente en el desarrollo de hongos fitopatógenos en los cereales [67]. En este sentido, Hay y colaboradores (2020) demostraron que altos niveles de CO_2 aumentan significativamente el crecimiento y la producción de DON en *F. graminearum* en trigo y, además, pueden incrementar la severidad de la enfermedad causada por este hongo [68].

- **Factores químicos**. El uso de compuestos fungicidas sigue siendo la principal estrategia para reducir la incidencia de hongos tanto en el campo como durante el almacenamiento [69]. El uso indiscriminado de estos compuestos ha causado un importante aumento del número de cepas resistentes, lo que hace muy difícil controlar eficazmente el crecimiento fúngico y el desarrollo de enfermedades en los cereales [70]. Además, estos compuestos no son biodegradables, por lo que pueden acumularse en el suelo, los acuíferos y las plantas, y en consecuencia contaminar el medio ambiente y afectar a los seres vivos. A todo esto se añade la tendencia mundial a reducir al mínimo el uso de productos químicos de síntesis en la cadena alimentaria. Estos compuestos pueden persistir en los alimentos, causando graves problemas para la salud humana y animal debido a que son carcinógenos, teratogénicos y provocan desequilibrios hormonales y espermatotoxicidad [70]. También cabe destacar que, en estas últimas décadas, la Unión Europea ha retirado del mercado una serie de productos fitosanitarios, lo que deja en evidencia la necesidad de desarrollar nuevos métodos alternativos más sostenibles y ecológicos para poder reducir el uso de fungicidas tradicionales. Los extractos de plantas pueden ser una buena alternativa a estos compuestos, ya que se ha visto que son seguros para el medio ambiente, al ser biodegradables y presentar baja toxicidad [71].

1.3. Legislación europea: niveles máximos de micotoxinas en los granos de cereal y sus subproductos

Como ya se ha mencionado anteriormente, las micotoxinas suponen un grave problema para la salud humana y animal. En lo que se refiere a seguridad alimentaria, el mayor riesgo se debe al consumo de cereales contaminados por micotoxinas [5]. Para controlar el contenido de micotoxinas en los alimentos y piensos, muchos países han establecido legislaciones con niveles máximos permitidos de algunas micotoxinas para salvaguardar la salud humana y animal. Estos niveles varían para cada toxina y alimento, y en función de si están o no procesados, estando los niveles más restrictivos en los productos infantiles. Esta normativa es dependiente de cada país, y no existe en la

actualidad una normativa para todas las toxinas conocidas ni aplicable a todas las matrices.

Desde el año 2001, la Unión Europea (UE) ha ido estableciendo diferentes normas para regular los niveles máximos permitidos de algunas micotoxinas en diferentes productos alimenticios destinados al consumo humano y animal. Dentro de este tipo de normativas están los reglamentos, que son actos jurídicos que se aplican de manera automática y uniforme en todos los países de la UE desde su entrada en vigor y, son obligatorios en los Estados miembros. Las recomendaciones por el contrario, pueden variar entre países y permiten a las instituciones de la UE dar a conocer sus puntos de vista y sugerir una línea de actuación sin imponer obligaciones legales [72].

A continuación, se muestra un resumen del contenido máximo para las principales micotoxinas que ha establecido la Comisión Europea (CE) en los cereales destinados a consumo humano desde el año 2006 hasta la fecha.

- **Aflatoxinas.** El contenido máximo de AF está regulado en el Reglamento Nº 165/2010 de la CE que modifica al Nº 1881/2006, en el que se dicta que la cantidad máxima de AFB_1 y para la suma de AFB_1+B_2+G_1+G_2 es de 2 y 4 μg/Kg, respectivamente, para todos los cereales y productos a base de cereales (incluidos los transformados). En el caso de maíz y arroz que vayan a someterse a un proceso de selección u otro tratamiento físico, antes del consumo humano directo o de su utilización como ingredientes de productos alimenticios, los límites se establecen en 5 μg/Kg de AFB_1 y 10 μg/Kg para la suma de AF, mientras que en alimentos infantiles y para lactantes no se pueden superan los 0,1 μg/Kg de AFB_1 [73].

- **Deoxinivalenol**. El contenido máximo de DON está regulado en el Reglamento Nº 1126/2007 de la CE que modifica al Nº 1881/2006, en el cual se fija la cantidad máxima de esta toxina en 1.750 μg/Kg para trigo duro, avena y maíz no elaborados (excepto el maíz destinado a molienda por vía húmeda) y 1.250 μg/Kg para el resto de los cereales no elaborados. En el caso de alimentos a base de cereales destinados al consumo humano directo es de 750 μg/Kg, excepto pan y aperitivos (500 μg/kg) y alimentos infantiles y para lactantes (200 μg/kg) [74].

- **Fumonisinas.** El contenido máximo de FB está regulado en el Reglamento Nº 1126/2007 que modifica al Nº 1881/2006, siendo la cantidad máxima de FB en el maíz no elaborado (excepto el destinado a molienda por vía húmeda) de 4.000 μg/Kg, y de 1.000 μg/Kg en alimentos a base de maíz destinados al consumo humano directo, excepto cereales y aperitivos (800 μg/Kg) y alimentos infantiles y para lactantes (200 μg/Kg) [74].

- **Ocratoxina A**. El contenido máximo de OTA está regulado en el Reglamento Nº 594/2012 que modifica al Nº1881/2006, en el cual se fija la cantidad máxima de OTA en cereales no elaborados (excepto los cereales destinado a molienda por vía húmeda) en 5 μg/Kg, y para los alimentos a base de cereales destinados al consumo humano directo es de 3 μg/Kg, excepto alimentos infantiles y para lactantes (0,5 μg/kg) [75].

- **Toxinas T-2 y HT-2.** El contenido de estas toxinas no está regulado por la UE, pero se ha publicado una recomendación, la Nº 165/2013/UE en la que se establece que los niveles de la suma de T-2+HT-2 en cereales no transformados no deberían superar los 1.000 μg/Kg para avena con cáscara, 200 μg/Kg para cebada, y 100 μg/Kg para trigo, centeno y otros cereales. Para los granos de cereal destinados al consumo humano la cantidad máxima recomendable es de 50 μg/Kg, excepto maíz (200 μg/Kg) y avena (100 μg/Kg). En alimentos a base de cereales destinados al consumo humano directo es de 200 μg/kg para el salvado y copos de avena, 100 μg/kg para los productos de molienda del maíz y 50 μg/kg para otros cereales, excepto pan y aperitivos (25 μg/kg) y alimentos infantiles y para lactantes (15 μg/kg) [76].

- **Zearalenona**. El contenido máximo de ZEA está regulado en el Reglamento Nº 1126/2007 que modifica al Nº 1881/2006, siendo la cantidad máxima de ZEA en maíz no elaborado (excepto el maíz destinado a molienda por vía húmeda) de 350 μg/Kg, y de 100 μg/Kg para el resto de los cereales no elaborados. En alimentos a base de cereales destinados al consumo humano directo es de 100 μg/Kg para el maíz y de 75 μg/Kg para el resto de los cereales, excepto pan y aperitivos (50 μg/kg) y alimentos infantiles y para lactantes (20 μg/kg) [74].

En lo referente a la alimentación animal, la UE legisla el contenido máximo de AFB_1, con límites entre 5-20 μg/kg, según el tipo de pienso (Directiva 2003/100/CE), y la recomendación Nº 2006/576/CE sobre la presencia de FB, DON, ZEA, OTA y toxinas T-2 y HT-2 en productos destinados a alimentación animal [6,77-79].

2. Prevención y control de micotoxinas

Una vez que las micotoxinas están presentes en los alimentos o los piensos, no se van a eliminar totalmente en los procesos normales de secado, molienda y procesado, ni en la cocción, debido a su alta estabilidad a los tratamientos físicos y químicos [12,28]. La detección temprana de especies potencialmente productoras de micotoxinas en los alimentos puede ser de ayuda para evitar que las micotoxinas entren en la cadena alimentaria. De esta manera, una vez que se conoce que un producto está contaminado con algún hongo toxígeno se podrían aplicar medidas correctivas adecuadas para evitar que se desarrolle y produzca micotoxinas [80]. Las técnicas moleculares basadas en protocolos de PCR específicos para cada especie son rápidas y permiten la identificación adecuada de las especies fúngicas debido a su alta especificidad y sensibilidad [81]. Es por ello que en las últimas décadas se han venido utilizando de manera rutinaria, adaptando el protocolo al tipo de matriz, y desarrollando nuevos protocolos que permiten la detección directa, y en algunos casos la cuantificación, de las especies productoras de micotoxinas más relevantes en el campo de la agroalimentación [82-87].

Las herramientas más destacadas para prevenir y controlar el crecimiento de los hongos toxígenos y la producción de micotoxinas en los cereales están basadas en la aplicación de buenas prácticas agrícolas (BPA) en el campo y el desarrollo de un plan de análisis de peligros y puntos de control crítico (APPCC) durante el procesado, almacenamiento y comercialización de los productos [88,89]. Como ya se ha mencionado

anteriormente, el crecimiento fúngico y la producción de micotoxinas pueden producirse durante todas las etapas de la cadena de producción, desde el desarrollo de las plantas en el campo y el almacenamiento de los granos, hasta el procesamiento industrial de los alimentos y piensos a base de cereales [90]. Es por ello que la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO) y la Organización Mundial de la Salud (OMS) recogieron en el *Codex Alimentarius* diferentes medidas de control, como los criterios de BPA y las pautas para establecer adecuados sistemas APPCC, con el fin de evitar la contaminación por micotoxinas, y garantizar la seguridad de los productos alimenticios para que lleguen al consumidor en condiciones óptimas [91].

Actualmente, se han desarrollado muchos métodos para controlar la contaminación por micotoxinas en los cereales y derivados, que se basan principalmente en la prevención del crecimiento fúngico tanto antes de la cosecha como durante el almacenaje [9]. A continuación, se detallan algunas de las medidas más significativas y las perspectivas para la aplicación de nuevos métodos de control.

2.1. Estrategias de control en campo

La mejor estrategia para evitar la presencia de micotoxinas en los alimentos es la prevención de su producción y acumulación en el campo. Establecer unas correctas BPA, un calendario de riego adecuado y elegir el óptimo momento de la cosecha son tres de los factores más determinantes a la hora de prevenir la contaminación por micotoxinas. Como ya se ha mencionado *Aspergillus* y *Fusarium* pueden crecer saprófitamente en los restos de la cosecha, por lo que un arado profundo, las rotaciones de cultivo y los barbechos van ayudar a prevenir la acumulación de residuos recalcitrantes en el suelo antes de la cosecha [60]. En lo que respecta al desarrollo vegetativo y reproductivo de los cereales, el riego es uno de los factores más importantes, ya que va a evitar el estrés hídrico que se genera en los cultivos de cereal en la época de sequía. Sin embargo, también puede contribuir a la diseminación de esporas e infección de plantas sanas, debido a las salpicaduras que se producen al regar que pueden ser un medio de transporte importante para las esporas de los hongos. Por este motivo es crucial no realizar riegos en la época de floración, donde las plantas de cereal son más susceptibles a ser infectadas por especies toxígenas de *Fusarium* [92]. Además, los riegos antes de la cosecha contribuyen al desarrollo fúngico durante la recolección y el almacenamiento, así que un periodo seco antes de la recogida es esencial para reducir los problemas de contaminación por micotoxinas [93].

A pesar de que se ha demostrado que estas estrategias ayudan a reducir la presencia de especies potencialmente toxígenas en los cereales, el método de control más extendido durante el desarrollo vegetativo y reproductivo de los cereales es la aplicación de fungicidas [54,60]. Los fungicidas basados en benzimidazoles, triazoles e isoxazoles son ampliamente utilizados para controlar el crecimiento de *Fusarium* y otros hongos en campo, pero su uso viene planteando un problema en estas últimas décadas. Estos compuestos persisten en los alimentos, amenazando la salud humana y animal, por lo que la búsqueda de nuevas alternativas más sostenibles está en auge [16,94-96,70].

Los metabolitos derivados de plantas, incluidos los compuestos fenólicos y los aceites esenciales (AE), son un sustituto prometedor de los fungicidas tradicionales, ya que abarcan una amplia variedad de compuestos, ya sean producidos como parte del desarrollo de la planta o como reacción al estrés o a los patógenos [97]. Diversos estudios describen que los fungicidas naturales obtenidos a partir de extractos de plantas tienen un gran potencial para controlar la acumulación de micotoxinas [98,99]. Los AE están formados por compuestos antimicrobianos y antioxidantes y se consideran una buena elección como fungicidas naturales, aunque los factores ambientales pueden afectar a su composición [100]. Las ventajas y desventajas de la utilización de los AE como agentes antifúngicos y antitoxígenos se tratan más adelante en el apartado 2.3.

Otra de las alternativas de protección sostenible de los cultivos es el uso de agentes de control biológico que impidan la proliferación de hongos toxígenos. Su efecto se basa en distintos mecanismos como el antagonismo directo entre el agente de control biológico y el hongo toxígeno, la competencia por la ocupación del nicho, y la producción de metabolitos secundarios o la producción de compuestos orgánicos volátiles que afecten al desarrollo fúngico [13]. Aunque la eficacia de distintos agentes de control biológico ha sido demostrada en condiciones de laboratorio, su comercialización depende de si pueden controlar de manera fiable los hongos toxígenos en campo. En Estados Unidos y África Occidental y Oriental se ha aplicado con éxito en cultivos de maíz utilizando una mezcla de cepas de *A. flavus* atoxígenas, para evitar el desarrollo de las cepas productoras de *A. flavus* y reducir así la contaminación por AFB_1 [101]. Otros estudios en campo han aplicado *Bacillus subtilis* y *Brevibacillus* sp. reduciendo la acumulación de DON en trigo [102].

La aparición de la nanotecnología y el rápido desarrollo de nanomateriales han hecho que en la actualidad, se estén aplicando estas técnicas para la producción de compuestos antifúngicos que, a su vez, sean capaces de inhibir la producción de micotoxinas [103-106]. El efecto de estos materiales puede ser debido a que se utilicen como vehículo de encapsulación de otros compuestos o a que los propios nanomateriales desempeñen directamente un papel clave en el efecto de inhibición de los hongos [9,106]. Varios de estos nanomateriales, como las nanopartículas metálicas (Fe_2O_3, SWCNT, CuNP y AgNP) están dando resultados muy prometedores como eficaces fungicidas contra hongos toxígenos [9]. Tarazona y colaboradores (2019) han demostrado en un reciente estudio la eficacia *in vitro* de nanopartículas (NP) de Ag frente a las principales especies toxígenas del género *Fusarium* y han obtenido buenos resultados para reducir tanto su crecimiento como su capacidad para producir toxinas [107]. Estos métodos de control son muy prometedores, aunque son necesarios más estudios para conocer el efecto de estas NP metálicas sobre la salud [9]. Para vencer este inconveniente ha surgido la nanobiotecnología, que desarrolla NP metálicas de baja toxicidad que incluyen compuestos naturales como los desechos agrícolas (coco, maíz, salvado de cereales, semillas y cascars de fruta, etc) [106, 108].

2.2. Estrategias de control durante el almacenamiento

La actividad de agua del grano, la temperatura, la incidencia de plagas y la contaminación fúngica son factores muy importantes durante el almacenamiento de los

granos de cereal [5,90]. Todos estos factores interactúan entre ellos para causar o evitar daños a los granos almacenados. La medida de control más importante para prevenir la contaminación por micotoxinas durante el almacenamiento de los cereales es reducir la a_w a menos de 0,70 y mantener este nivel durante todo el período de almacenamiento [60], ya que, de esta manera, los hongos no pueden desarrollarse en los granos [109]. A su vez, es importante controlar que el grano se mantenga a baja temperatura para limitar su respiración, y de este modo evitar que se genere agua, que pudiera modificar el contenido de humedad [59]. Además, a baja temperatura se reduce considerablemente el crecimiento fúngico y su capacidad para sintetizar las toxinas [96]. También es importante mantener una temperatura constante, ya que así hay menos probabilidad de que se generen bolsas de condensación en el interior del silo que afectarían drásticamente a la humedad ambiental [110].

El control de las plagas durante el almacenamiento también es imprescindible, no solo por las pérdidas que generan al reducir la calidad nutricional del grano, sino porque pueden afectar significativamente al desarrollo de los hongos toxígenos, ya que la respiración de los insectos puede aumentar las condiciones de humedad en el grano a niveles no seguros. Además, el grano dañado por estos organismos es más susceptible a la colonización de los hongos y a su vez, los insectos pueden actuar como vectores diseminando las esporas por el silo [54].

Algunos ácidos orgánicos como por ejemplo los ácidos propiónico ($C_3H_6O_2$), acético ($C_2H_4O_2$) y fórmico (CH_2O_2), así como sus sales, son utilizados durante el almacenamiento de los cereales para reducir tanto el crecimiento fúngico como la aparición de insectos [54]. Como ya se ha mencionado anteriormente, el uso de estos compuestos es perjudicial para el medioambiente y puede afectar a la salud de los consumidores. Además son compuestos corrosivos por lo que se requiere de equipos de protección individual para su aplicación, y además pueden generar pérdidas en la calidad del grano [16].

La búsqueda de métodos alternativos para la conservación de los granos en el almacenamiento está en auge y en estas últimas décadas se han evaluado diferentes agentes como el ozono, los antioxidantes y los AE [16,96,111]. Varios estudios *in vitro* han confirmado una reducción en el contenido de micotoxinas tras aplicar antioxidantes (butilhidroxianisol, propilparabeno o resveratrol) o extractos de plantas aromáticas en los cereales [112,113]. Sin embargo, son necesarios estudios más amplios para solventar sus limitaciones y hacer de estos agentes una estrategia de control fiable para la agricultura.

2.3. Uso de extractos naturales para el control de hongos toxígenos

Como ya se ha mencionado anteriormente, los compuestos fungicidas convencionales son muy eficaces y se han utilizado durante décadas para prevenir el desarrollo de los hongos [69]. Sin embargo, la preocupación de los consumidores por la seguridad de los alimentos y el cuidado del medioambiente, así como la restricción del uso de algunos de los estos fungicidas [114-116], ha propiciado la necesidad de desarrollar nuevos agentes entre los que destacan los extractos de plantas.

Los extractos de plantas aromáticas han demostrado tener fuertes propiedades antibacterianas, antifúngicas e insecticidas. Estas cualidades junto con su baja toxicidad, y su aceptación por parte de los consumidores, los hace buenos candidatos como fungicidas naturales, en sustitución de los productos tradicionales [117]. Se ha demostrado que estos extractos son ricos en compuestos potencialmente bioactivos, como las fitoalexinas, los alcaloides, flavonoides, isoflavonoides, taninos, cumarinas, glucósidos, terpenos, fenilpropanos y ácidos orgánicos para la protección de las plantas [118-120]. Además se ha descrito que no solo reducen el crecimiento fúngico, sino que son capaces también de interferir en la biosíntesis de las micotoxinas [121,122]. El hecho de que estén constituidos por una gran variedad de compuestos activos de estructura muy diferente, les confiere la ventaja de tener un amplio espectro de acción y dificulta el desarrollo de resistencias [70,117].

Por lo tanto, el uso de estos extractos naturales para prevenir el crecimiento fúngico durante el almacenamiento de los cereales, podría ser una solución sostenible para reducir al mínimo las pérdidas de alimentos debido a la contaminación por micotoxinas [123]. Sin embargo los AE tienen el inconveniente de ser compuestos volátiles que se degradan con facilidad en presencia de la luz solar, el aire o el calor. Además, su aplicación directa durante el almacenamiento puede ser limitada, no solo por su alta volatilidad, sino también por su baja solubilidad en agua y alta susceptibilidad a la oxidación [124]. Para solventar estos problemas es necesario desarrollar un correcto método de aplicación para preservar sus propiedades. La encapsulación de AE ofrece numerosas ventajas tales como prolongar la actividad de sus compuestos activos, mejorar su estabilidad a la oxidación, una liberación más controlada y una mayor facilidad de manejo, lo que conlleva a un mayor rendimiento de sus propiedades antifúngicas y antitoxígenas [125]. Además, los AE encapsulados son compuestos biodegradables que presentan una baja toxicidad y pueden almacenarse con facilidad, lo que constituye una importante ventaja para su aplicación en los almacenes de grano. Existen diversas técnicas de encapsulación, siendo las más comunes el secado por pulverización o atomización, la pulverización por enfriamiento, la extrusión, la encapsulación en alginato de sodio, la coacervación y los sistemas basados en lípidos (liposomas y niosomas). La elección de uno u otro método va a depender del tipo de aplicación que se vaya a realizar, las propiedades físicas y químicas del compuesto a encapsular, así como de la escala de producción y su coste [124,126].

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OBJETIVOS

Los cereales son una fuente básica de la alimentación a nivel mundial, tanto humana como animal, por lo que su contaminación por hongos toxígenos y sus micotoxinas suponen una grave amenaza para la seguridad alimentaria. La detección temprana de dichas especies así como la búsqueda de nuevos métodos sostenibles de control para reducir la incidencia de micotoxinas en los cereales es de vital importancia. Por todo ello, se definieron los siguientes objetivos en la presente Tesis Doctoral:

Objetivo 1. Evaluar a largo del ciclo de cultivo del maíz la contaminación por micotoxinas y sus hongos productores.

Objetivo 2. Estudiar la presencia de las principales especies toxígenas de los géneros *Fusarium* y *Aspergillus* en maíz y avena recolectados en campo en las principales zonas cerealistas españolas, así como en muestras comerciales de productos derivados de los mismos consumidas en España.

Objetivo 3. Evaluar el efecto *in vitro* de aceites esenciales y sus correspondientes hidrolatos sobre el crecimiento de *Aspergillus flavus* y su capacidad para producir aflatoxinas.

Objetivo 4. Evaluar el efecto *in vitro* de los compuestos naturales seleccionados en el objetivo 4 bajo diferentes condiciones de actividad de agua sobre el crecimiento temprano y la producción de aflatoxinas en *Aspergillus flavus*.

Objetivo 5. Desarrollar un sistema eficaz para la aplicación de los aceites esenciales seleccionados durante el almacenamiento de los granos de maíz.

CHAPTER 1

A comprehensive study on the occurrence of mycotoxins and their producing fungi during the maize production cycle in Spain.

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Abstract

Mycotoxin contamination is one of the main problems affecting corn production, due to its significant risk to human and animal health. The *Fusarium* and *Aspergillus* species are the main producers of mycotoxins in maize, infecting both pre-harvest and during storage. In this work, we evaluated the presence of mycotoxins and their producing species along maize production cycles in three different stages (anthesis, harvest, and storage) during three consecutive seasons (2016–2018). Fungal occurrences were studied using species-specific PCR protocols, whereas mycotoxin levels were determined by LC-MS/MS. Fumonisin-producing *Fusarium* species (*F. verticillioides* and *F. proliferatum*), as well as the aflatoxin producer *Aspergillus flavus*, were the most predominant species at all stages; although, during some seasons, the presence of *F. graminearum* and *A. niger* aggregate species were also identified. Contrastingly, fumonisins were the only mycotoxins detected and levels were always under legal regulations. The results presented here demonstrate that even when fungal contamination occurs at the early stages of the maize production cycle, the application of good agricultural and storage practices might be crucial to ensure mycotoxin-free grains.

Keywords

Aflatoxins; fumonisins; good agricultural practices; corn; PCR-detection; *Aspergillus*; *Fusarium.*

1. Introduction

Maize (*Zea mays* L.) is a monoecious plant of the Poaceae family, and it is cultivated worldwide. Two phases can be distinguished during its growth—vegetative and reproductive. The vegetative phase begins with the growth of the plant and culminates with the appearance of the male flower. The reproductive phase begins with the emergence of the female flower, and the physiological maturity of the cob takes place in this stage [1]. Maize is one of the most important cereals, with an annual worldwide production of 1134 million tons in 2017, according to the Food and Agriculture Organization of the United Nations [2].

One of the main problems regarding corn production is mycotoxin contamination. Mycotoxins are toxic secondary metabolites, produced by several fungi, that frequently contaminate maize in the field and/or during storage [3]. The most relevant fungal genera affecting maize are *Aspergillus* and *Fusarium* [4]. The main mycotoxins associated with corn during all of its production cycles and its storage are fumonisins (FUMs), trichothecenes (TCTs), zearalenone (ZEA), aflatoxins (AFs), and ochratoxin A (OTA) [5].

Maize grains are often contaminated simultaneously with various mycotoxin-producing species, the most relevant being *Fusarium verticillioides* and *F. proliferatum*, the main FUM-producing species; *F. graminearum*, which produces TCTs and ZEA; and *Aspergillus flavus*, the main AF-producing species [6].

The consumption of corn contaminated by mycotoxins may cause a number of severe toxic effects in both animals and humans. The International Agency for Research on Cancer (IARC) has classified AFB_1 as a carcinogen to humans (group 1) and fumonisin B_1 (FB_1) and OTA as possible human carcinogens (group 2B) [7]. Due to the health risk associated with the intake of foodstuffs contaminated by mycotoxins, the European Union has established—through legislation—the maximum levels of certain toxins allowed in maize and its derived products. The European Commission (CE) Regulation N° 1881/2006 [8] set the maximum levels for AFs, FUMs, ZEA, and Deoxynivalenol (type B TCT) in unprocessed maize and different maize-derived products intended for human consumption. This regulation was subsequently modified by CE N° 1126/2007 [9] and CE N° 165/2010 [10] to apply more restrictions on the level of AFs and *Fusarium* toxins allowed in these products. Currently, there is no legislation setting the maximum levels of T-2 and HT-2 (type A TCTs) or OTA in corn, due to a lack of studies regarding their relevance in this food matrix.

The presence of these toxins, apart from constituting a threat to human and animal health, may cause serious economic losses to farmers, who have to discard contaminated grains [11]. Moreover, feed contamination by mycotoxins also increases veterinary care costs and severely reduces livestock production [11].

Traditionally, the species included in the *Fusarium* genus are considered field fungi, although FUM production often happens during post-harvest, when corn storage conditions are inadequate and permissive for toxin production [12]. On the other hand, *A. flavus*—which has been traditionally considered a storage fungus—can infect maize both pre-harvest and during storage, and an increase in AF content is

likely if the drying and storage conditions are not appropriate [6]. However, there are not currently enough data to support this statement. Therefore, it is essential to know what happens during the maize production cycle and to establish adequate control methods to avoid mycotoxins entering the food chain.

Mycotoxins are introduced into food chains by the pre- or post-harvest contamination of food and feed [13]. The early detection of toxin-producing species contaminating maize is one of the most important actions in preventing mycotoxin contamination [14]. The identification of these fungal species has been traditionally performed using traditional isolation and culturing techniques [15]. However, these conventional methods for the identification and detection of these fungi in food and feed are time consuming and require taxonomical expertise [16]. Hence, it is important to develop fast and reliable techniques to detect mycotoxin producers within foodstuffs. PCR-based protocols that target DNA are considered a good alternative for rapid diagnosis, due to their high specificity and sensitivity and have been used for the detection of toxigenic species in maize and maize products [17]. The main advantages of species-specific PCR protocols are that they offer a sensitive, high-throughput method for identifying fungi in complex mixtures, even when they are no longer viable [18]. It is not necessary to culture the organisms for a long time, only 24–48 h before detection—which significantly reduces the time of the analysis [15,16].

The aim of this study is to evaluate, for the first time, the presence of mycotoxin-producing species along maize production cycles at three different stages (anthesis, pre-harvest, and storage) and its possible relationship with mycotoxin contamination. For this purpose, we analyze the samples with previously optimized PCR protocols or new ones developed in this work. Subsequently, mycotoxin levels are detected by LC-MS/MS.

2. Materials and Methods

2.1. Corn samples and fungal isolates

2.1.1. Corn samples

Maize samples of different varieties (DKC 6630, DKC 6442, and DKC 6728) were collected from a farm located in the South Area of the Community of Madrid (Spain), at three crop stages (anthesis, pre-harvest, and storage), along three consecutive seasons (2016, 2017, and 2018). In this farm, good agricultural practices (GAP) and adequate fallow periods are correctly applied. The study was performed sampling different continuous plots (P); two plots were sampled in 2016 (P1-P2) whereas three different plots were evaluated in 2017 (P1-P2-P3) and 2018 (P2-P3-P4). A total of 27 samples were evaluated (Table 1).

Table 1. Characteristics of maize samples analyzed in this study, obtained from three growth stages during three consecutive seasons.

Season	Stage	Plot	Variety	Sample Code
2016	ANTHESIS-MALE	P1	DKC 6630	P1.FM-16
		P2	DKC 6442	P2.FM-16
	ANTHESIS-FEMALE	P1	DKC 6630	P1.FF-16
		P2	DKC 6442	P2.FF-16
	PRE-HARVEST	P1	DKC 6630	P1.PRE-16
		P2	DKC 6442	P2.PRE-16
	STORAGE			SILO-16
2017	ANTHESIS-MALE	P1	DKC 6442	P1.FM-17
		P2	DKC 6442	P2.FM-17
		P3	DKC 6630	P3.FM-17
	ANTHESIS-FEMALE	P1	DKC 6442	P1.FF-17
		P2	DKC 6442	P2.FF-17
		P3	DKC 6630	P3.FF-17
	PRE-HARVEST	P1	DKC 6442	P1.PRE-17
		P2	DKC 6442	P2.PRE-17
		P3	DKC 6630	P3.PRE-17
	STORAGE			SILO-17
2018	ANTHESIS-MALE	P2	DKC 6630	P2.FM-18
		P3	DKC 6728	P3.FM-18
		P4	DKC 6442	P4.FM-18
	ANTHESIS-FEMALE	P2	DKC 6630	P2.FF-18
		P3	DKC 6728	P3.FF-18
		P4	DKC 6442	P4.FF-18
	PRE-HARVEST	P2	DKC 6630	P2.PRE-18
		P3	DKC 6728	P3.PRE-18
		P4	DKC 6442	P4.PRE-18
	STORAGE			SILO-18

P (plot); MF (male flower); FF (female flower); PRE (pre-harvest)

The sampling procedure for each plot was set as follows: (1) in the anthesis period, 25 male flowers (MF) and 25 female flowers (FF) were collected; (2) during pre-harvest (approximately 7 days before harvest), 25 cobs (PRE) (grain moisture 35 %) were sampled; (3) after three months of storage, 3 kg of corn grain from different points of the silo were collected. The relative humidity and temperature were registered every 8 h during silo storage in 2018 using a data logger El-USB-1 (LASCAR electronic, Salisbury, UK).

For each plot, the samples were collected at random, taking one sample every 3 m along the plot. When they arrived at the laboratory, all the samples were separated into three lots. Subsequently, flower samples, cobs of pre-harvest (previously threshed), and silo samples were crushed with an IKA A11 Basic Mill (IKA, Königswinter, Germany) to obtain a fine powder, according to European Union requirements [19]. The milled samples were placed in hermetic sterile plastic bags and stored at −20 °C until analysis. All samples were analyzed in triplicate.

2.1.2. Fungal isolates and culture conditions

All the isolates used in this study to optimize species-specific PCR protocols—along with their sources—are listed in Table 2. The fungal strains came from different culture collections or they were isolated in our laboratory from Spanish cereal samples.

The strains were maintained by regular sub-culturing on potato dextrose agar medium (PDA) (Pronadisa, Madrid, Spain) at 25 ± 1 °C for 5 days and stored as spore suspensions in 15 % glycerol (Panreac, Madrid, Spain) at −80 °C until required.

Table 2. Fungal strains analyzed indicating the origin, species, accession number, and presence (+) or absence (-) of the specific PCR amplification product of *F. temperatum* (FT), *F. langsethiae* (FL) *F. sporotrichioides* (FS), *F. poae* (FP), *F. graminearum* (FG), *and F. fujikuroi* (FJ) using the specific protocols designed in this study.

STRAIN	ORIGIN	SPECIES	ACCESS NUMBER	FT	FL	FS	FP	FG	FF
ITEM 550[a]	Poland	*F. sporotrichioides*		-	-	+	-	-	-
ITEM 695[a]	USA	*F. sporotrichioides*		-	-	+	-	-	-
ITEM 707[a]	Poland	*F. sporotrichioides*		-	-	+	-	-	-
ITEM 1442[a]		*F. sporotrichioides*		-	-	+	-	-	-
ITEM 4596[a]	Russian	*F. sporotrichioides*		-	-	+	-	-	-
ITEM 4597[a]	Russian	*F. sporotrichioides*		-	-	+	-	-	-
CECT 20150[b]		*F. sporotrichioides*		-	-	+	-	-	-
CECT 20166[b]	Russian	*F. sporotrichioides*		-	-	+	-	-	-
ITEM 6606[a]	England	*F. poae*		-	-	-	+	-	-
ITEM 6607[a]	England	*F. poae*		-	-	-	+	-	-
MUCL 6114[c]	Belgium	*F. poae*		-	-	-	+	-	-
MUCL 7555[c]	Belgium	*F. poae*		-	-	-	+	-	-
MUCL 42824[c]	Belgium	*F. poae*		-	-	-	+	-	-
CBS 747.97[e]	USA	*F. subglutinans*	MN861787	-	-	-	-	-	-
MPE-0990[d]		*F. subglutinans*	MN861796	-	-	-	-	-	-
CBS 138.287[e]	Mexico	*F. temperatum*	MN861786	+	-	-	-	-	-
F1-VERT	Spain	*F. verticillioides*	MN861741	-	-	-	-	-	-
F3-VERT	Spain	*F. verticillioides*	MN861743	-	-	-	-	-	-
MPA 0999[d]	USA	*F. verticillioides*	MN861799	-	-	-	-	-	-
F2-PRO	Spain	*F. proliferatum*	MN861742	-	-	-	-	-	-
F4-PRO	Spain	*F. proliferatum*	MN861745	-	-	-	-	-	-
MPD 4853[d]		*F. proliferatum*	MN861797	-	-	-	-	-	-
ITEM 4092[a]	Italy	*F. fujikuroi*	MN861805	-	-	-	-	-	+
ITEM 4093[a]	Italy	*F. fujikuroi*		-	-	-	-	-	+
ITEM 4094[a]	Italy	*F. fujikuroi*		-	-	-	-	-	+
ITEM 4095[a]	Italy	*F. fujikuroi*		-	-	-	-	-	+
MPC1993[d]		*F. fujikuroi*		-	-	-	-	-	+

Cont. Table 2.

MPC1995[d]	Taiwan	*F. fujikuroi*		-	-	-	-	-	+
PRC 14a[h]		*F. fujikuroi*		-	-	-	-	-	+
PRC 19a[h]		*F. fujikuroi*		-	-	-	-	-	+
ITEM 6013[a]	USA	*F. globosum*	MN861806	-	-	-	-	-	-
ITEM 1590[a]	Italy	*F. globosum*	MN861808	-	-	-	-	-	-
F23-LANG	Spain	*F. langsethiae*	MN861761	-	+	-	-	-	-
L.3.1		*F. langsethiae*		-	+	-	-	-	-
L.3.2		*F. langsethiae*		-	+	-	-	-	-
NRRL 28585[f]	Venezuela	*F. graminearum*		-	-	-	-	+	-
NRRL 28436[f]	New Caledonia	*F. graminearum*		-	-	-	-	+	-
NRRL 29020[f]	South Africa	*F. graminearum*		-	-	-	-	+	-
NRRL 29148[f]	USA	*F. graminearum*		-	-	-	-	+	-
NRRL 26755[f]	South Africa	*F. graminearum*		-	-	-	-	+	-
NRRL 13818[f]	Japan	*F. graminearum*		-	-	-	-	+	-
NRRL 29169[f]	USA	*F. graminearum*		-	-	-	-	+	-
NRRL 28585[f]	New Zealand	*F. graminearum*		-	-	-	-	+	-
ITEM 628[a]	Yugoslavia	*F. culmorum*		-	-	-	-	-	-
ITEM 4335[a]		*F. culmorum*		-	-	-	-	-	-
ITEM 6717[a]	Hungary	*F. culmorum*		-	-	-	-	-	-
ITEM 6718[a]	Hungary	*F. culmorum*		-	-	-	-	-	-
Be1:H3-1/1G	Spain	*F. avenaceum*		-	-	-	-	-	-
ITEM 4094[a]		*F. thapsinum*	MN861807	-	-	-	-	-	-
MPB 3852[d]		*F. sacchari*	MN861798	-	-	-	-	-	-
VI01093[g]	Norway	*F. equiseti*		-	-	-	-	-	-
F100	Spain	*F. oxisporum*	MN861795	-	-	-	-	-	-
F103	Spain	*F. solani*	MN861794	-	-	-	-	-	-

Strains retrieved from [a] Agrofood Microbial Culture Collection (ISPA, Bari, Italy), [b] Spanish Type Culture Collection (CECT, Valencia, Spain), [c] Belgian Coordinated Collections of Micro-organisms (BCCM, Brussels, Belgium), [d] Hurbarium Collections at the Charles University (PRC, Prague, Czechia), [e] Westerdijk Fungal Biodiversity Institute Collection (CBS-KNAW, Utrecht, The Netherlands), [f] Agricultural Research Center Culture Collection (USDA, Washington, DC, USA), [g] Strain from *G. fujikuroi* mating populations A-H.

2.2. Primer design and PCR amplification

Specific primer sets were designed on the basis of sequence alignments of the partial region of the elongation factor 1α (*tef-1*α) encoding gene. For the alignments, sequences of more than twenty strains from different origins were used. The alignments included sequences from the *Fusarium* species, as well as from other related species obtained in this paper, or in previous works carried out in our laboratory, or retrieved from the NCBI database. The sequences were edited and aligned using the ClustalW method, using UGENE 1.29 software (Unipro, Novosibirsk, Russia).

Genomic DNA extraction from *Fusarium* strains listed in Table 2 was carried out using the protocol described elsewhere [20]. Fungal mycelia from four-day-old cultures on PDA plates were scraped off with a scalpel, frozen with liquid nitrogen and ground using a micropistille prior to DNA extraction. DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, NC, USA). All PCR assays were performed in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). Genomic DNAs were amplified using primers EF-1 and EF-2 [21], which amplify a partial region of the *tef-1α* gene. The amplification reactions were carried out in volumes of 25 μL, containing 100 ng of sample DNA, 1 μL of each primer (20 μM; Metabion, Planegg, Germany) and 12.5 μL of NZYTaq II 2x Green Master Mix (Nzytech, Lisbon, Portugal).

PCR products were detected in 2 % agarose ethidium bromide gels in TAE 1X buffer (Tris Acetate 40 mM and EDTA 1.0 mM). The NZYDNA Ladder V (Nzytech, Lisbon, Portugal) was used as the molecular size marker.

The amplification products approximately 670 bp-long were purified using the NZYGelpure Kit (Nzytech, Lisbon, Portugal) and sequenced with an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA), according to manufacturer's instructions in Macrogen facilities (Madrid, Spain). All amplification products were sequenced in both directions.

The sequences were assembled using the UGENE 1.29 package. The sequences were compared with those deposited on NCBI nucleotide databases to reach the identification of the corresponding isolates at species level. Subsequently, these sequences were deposited into the NCBI database.

Specific PCR assay of *F. graminearum* was carried out using primers GRAM.EF-F (5′-AACCCCGCCGACACTTGGCG-3′) and GRAM.EF-R (5′-GGTTGACACGTGATGATGAGCG-3′), and the following protocol: 1 cycle of 5 min at 94 °C, 30 cycles of 35 s at 95 °C (denaturation), 45 s at 66 °C (annealing), 30 s at 72 °C (extension) and, finally, 1 cycle of 5 min at 72 °C, was followed.

In the case of *F. langsethiae*, the primers were LANG.EF-F (5′-GCTCTTCCTTCCCACATAGCCA-3′) and LANG.EF-R (5′-GCAGGCATGTTAGTATGATAATG-3′) and the protocol was: 1 cycle of 5 min at 94 °C, 28 cycles of 35 s at 95 °C, 20 s at 62 °C, 30 s at 72 °C and, finally, 1 cycle of 5 min at 72 °C.

For *F. fujikuroi*-specific detection, the primers designed were FUJI.EF.F (5′-TTGCCCACCGATTTCCCTTACGAT-3′) and FUJI.EF.R (5′-GTTAGTATGAATAAGTAGAATGAAGCAT-3′), and the protocol was: 1 cycle of 5 min at 95 °C, 30 cycles of 35 s at 95 °C, 30 s at 61 °C, 30 s at 72 °C and, finally, 1 cycle of 5 min at 72 °C.

The primer set POAE.EF-F (5′-GCATTTCTTTGGGCGCGAATCG-3′) and POAE.EF-R (5′-TGAGTGACTGAGGTAGTAGTGAC-3′) was used in the case of *F. poae*, using the protocol: 1 cycle of 5 min at 94 °C, 30 cycles of 35 s at 95 °C, 20 s at 66 °C, 30 s at 72 °C and, finally, 1 cycle of 5 min at 72 °C.

A specific PCR assay of *F. sporotrichioides* was performed using the primers SPORO.EF-F (5′-GCTTTTGCCCTTCCCACACAT-3′) and SPORO.EF-R (5′-AATGTGATGAAGGCAATAGTGAC-3′), and the protocol: 1 cycle of 5 min at 94 °C, 30 cycles of 35 s at 95 °C, 20 s at 62 °C, 30 s at 72 °C and, finally, 1 cycle of 5 min at 72 °C.

Finally, in the case of *F. temperatum* the primers designed were TEMP.EF-F (5′-CAAGACCTGGCGGGCATCTCA-3′) and TEMP.EF-R (5′-CTCAGAAGGTTGTGGCAATGG-3′) and the protocol: 1 cycle of 5 min at 95 °C, 27 cycles of 30 s at 95 °C, 30 s at 64 °C, 25 s at 72 °C and finally 1 cycle of 5 min at 72 °C.

2.3. Study on the occurrence of mycotoxins and mycotoxin-producing fungi on maize samples

2.3.1. PCR detection of the main mycotoxin-producing *Fusarium* and *Aspergillus* species

2.3.1.1. DNA extraction

Before DNA extraction, 1 g of the milled sample was cultured in 250 mL Erlenmeyer flasks containing 50 mL Sabouraud–Chloramphenicol broth (Pronadisa, Madrid, Spain), at 28 °C on an orbital shaker SK-0330-PRO (140 rpm) (Labolan, Navarra, Spain) in darkness for 24 h. Subsequently, the cultures were filtered through Whatman N° 1 paper. Filtered cultures were frozen with liquid nitrogen and ground using a mortar and pestle. The samples were kept at −80 °C until DNA extraction.

For each sample, DNA extraction was carried out in triplicate using the NZYPlant/Fungi gDNA Isolation Kit (Nzytech, Lisbon, Portugal) according to the manufacturer's instructions (DNA extraction protocol from fungi). DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, NC, USA).

2.3.1.2. Detection *Aspergillus* and *Fusarium* species, by specific PCR assays

Species-specific PCR protocols were applied to detect the aflatoxin (AF) producers *A. flavus* [22] and *A. parasiticus* [23]. The presence of the most relevant ochratoxin A (OTA) producers (*Aspergillus carbonarius*, *A. westerdijkiae*, *A. ochraceus* and *A. steynii*) was also evaluated using protocols previously described in our laboratory [24,25]. Some *A. niger* aggregate species (*A. niger* and *A. welwitschiae*) are able to produce both OTA and fumonisin B_2 (FB_2), and their presence was also tested using the specific protocols described by Palumbo [26].

The presence of the fumonisin-producing *Fusarium* species was also tested using species-specific PCR protocols. Some assays are described in the present work (*F. temperatum* and *F. fujikuroi*), whereas other protocols were previously described by our group for their use in detecting *F. proliferatum* [27] and *F. verticillioides* [28]. The specific primer pair for *F. subglutinans* was designed by Scauflaire et al. (2012) [29],

although it was used with the amplification protocol described for *F. temperatum* (Section 2.2). PCR protocols were also applied to detect the trichothecenes (TCT)- and zearalenone (ZEA)-producing *Fusarium* species. *F. graminearum* was evaluated using the protocol described here, whereas *F. equiseti* and *F. culmorum* were detected by the protocol described in Jurado et al. (2005) [17]. The specific detection of the type A TCT-producing species *F. poae*, *F. sporotrichioides* and *F. langsethiae* was performed using the protocols described in this article.

The PCR assays and the detection of the PCR products were carried out as described in the Section 2.2 *(*primer design and PCR amplification). Before the test using specific protocols, the presence of fungal DNA in all samples was confirmed using universal primers ITS1/ITS4 [30].

2.3.2. Mycotoxin determination

Mycotoxin analyses were performed in the "Laboratorio Arbitral Agroalimentario" (Madrid, Spain) following its standardized protocols. The presence of AFB_1, OTA, fumonisins B_1 and B_2 (FB_1 and FB_2), TCT type A (T-2 and HT-2 toxins) and type B (deoxynivalenol (DON)) and ZEA were analyzed in the pre-harvest and stored maize samples.

To determine the toxin concentrations of the samples, 5 g of dry milled maize was thoroughly mixed with 25 mL acetonitrile/water/formic acid (79:20:1, *v*/*v*) in polypropylene tubes. The mixture was shaken in an orbital shaker (Excella® E24, New Brunswick Scientific, Eppendorf, Germany) for 30 min to extract toxins, and then centrifuged at 5000 rpm for 5 min. Subsequently, 500 μL of extract were collected in a vial, then 25 μL of internal standards solution was added. The solvent was evaporated in a gentle stream of N_2 at 50 °C, and the residue was solved in 250 μL of mobile phase B, then 250 μL of mobile phase A was added. The extracts were filtered using cellulose syringe filter, with a 0.22 μm pore size (Minisart®, Sartorius Stedim, Germany) and then were transferred into vials and stored at −20 °C until analysis. All samples were performed by duplicate.

The samples were examined by LC-MS/MS using a 325 LC/MS system (Varian Inc., Palo Alto, CA, USA), equipped with an ESI interface and HPLC system, with a 212 LC binary pump and a 460 LC automatic microinjector from Agilent Technologies (Waldbronn, Germany).

Separation was performed on an EC-C18 column (Poroshell 120 Agilent 50 × 4.6 mm, 2.7 μm particle size). The column temperature was 25 °C. The injection volume was 20 μL. The mobile phase consisted of a mixture of 0.15 % formic acid with 0.5 mM ammonium formate in water (A) and 0.1 % formic acid in methanol (B) and the gradient was programmed as follows: 0.00 min; 90 % A, 10 % B. 1.42 min; 90 % A, 10 % B. 2.54 min; 60 % A, 40 % B. 15.06 min; 35 % A, 65 % B. 15.30 min; 0 % A, 100 % B. 22.00 min; 0 % A, 100 % B. 22.54 min; 90 % A, 10 % B. 30.00 min; 90 % A, 10 % B. The flow-rate was 250 μL/min.

Standard curves were constructed with different levels of mycotoxin standards (Biopure™, Romerlabs, Tulln an der Donau, Austria). The detection limits were of 2 μg/kg in the case of AFB_1, 6 μg/kg for OTA, 180 μg/kg for FB_1, 60 μg/kg for FB_2, 6 μg/kg for T-2, 8 μg/kg for HT-2, 80 μg/kg for DON, and 12 μg/kg for ZEA.

3. Results

3.1. Optimization of species-specific PCR protocols

The sequences of the partial region of the *tef-1α* gene were obtained from several *Fusarium* strains and deposited on the NCBI database with accession numbers between MN861741 and MN861808. Some *tef-1α* sequences already available on databases were also included in the alignments. On the basis of these alignments, species-specific primers and PCR protocols were designed in order to detect *Fusarium graminearum*, *F. langsethiae*, *F. fujikuroi*, *F. poae, F. sporotrichioides* and *F. temperatum.* The specificity of the PCR assays developed was tested using DNA isolated from a wide range of fungal strains (Table 2).

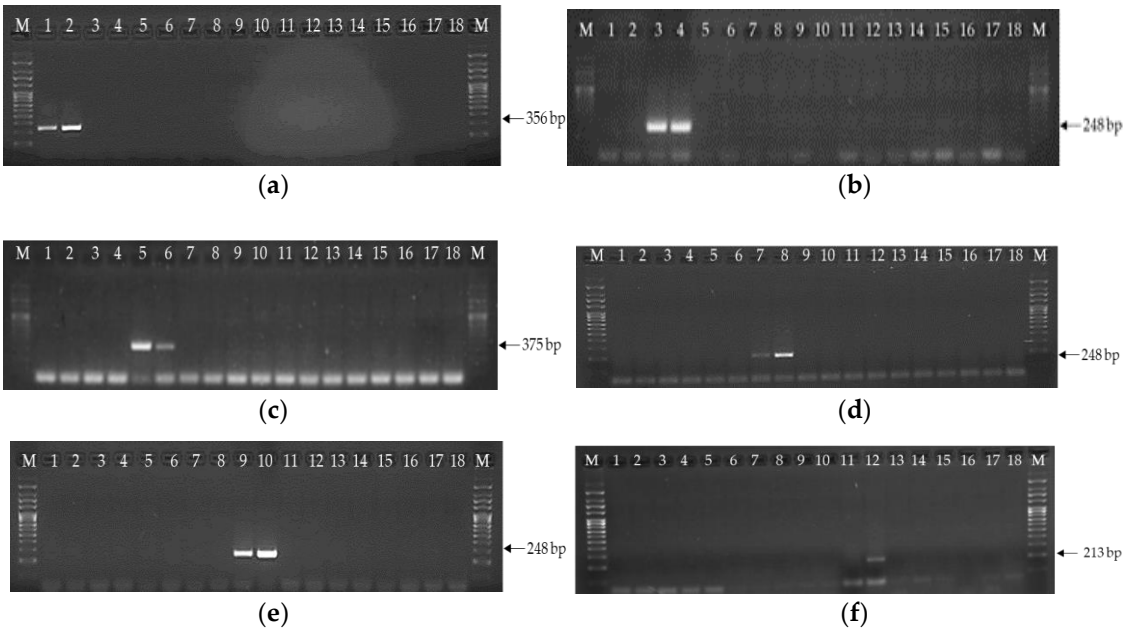

Figure 1. PCR amplification using the optimized protocols for specific detection of *F. graminearum* (**a**) *F. langshetiae* (**b**), *F. fujikuroi* (**c**) *F. poae* (**d**), *F. sporotrichioides* (**e**) and *F. temperatum* (**f**). Lanes 1 and 2: *F. graminearum* NRRL 28585, NRRL 28436; Lanes 3 and 4: *F. langsethiae* L.3.1, L.3.2*;* Lanes 5 and 6: *F. fujikuroi* ITEM 4092, ITEM 4093; Lanes 7 and 8: *F. poae* ITEM 6006, ITEM 6007; Lanes 9 and 10: *F. sporotrichioides* ITEM 550, ITEM 695; Lane 11: *F. subglutinans* CBS 747.97; Lane 12: *F. temperatum* CBS 138.287; Lane 13: *F. culmorum* ITEM 628; Lane 14: *F. globosum* ITEM 613; Lane 15: *F. equiseti* VIo1093; Lane 16: *F. verticillioides* F1-VERT; Lane 17: *F. proliferatum* F2-PRO; Lane 18: non template control. M: DNA molecular size 1000 bp marker.

As an example, Figure 1 shows the agarose gel electrophoreses and the results after the application of the species-specific PCR protocols designed using DNA from relevant *Fusarium* species. Figure 1a shows the results using GRAM.EF-F/GRAM.EF-R primers and their specific protocol, which amplified a single fragment of 356 bp solely

when the genomic DNA of *F. graminearum* strains was used. Figure 1b,d, and e show the results of specific assays which used LANG.EF-F/LANG.EF-R, POAE.EF-F/POAE.EF-R and SPOR.EF-F/SPOR.EF-R primer sets, respectively, which amplified a single fragment of 248 bp, when genomic DNAs of *F. langsethiae, F. poae* or *F. sporotrichioides* strains were used. Similarly, the PCR amplifications of genomic DNA from all the isolates indicated in Table 2 were performed using primers FUJI.EF-F/FUJI.EF-R and TEMP.EF-F/TEMP.EF-R and their corresponding amplification protocols. In the first case, a single fragment of 375 bp was only obtained when genomic DNA was used from either *F. fujikuroi* (Figure 1c), whereas using the second protocol, a fragment of 213 bp was observed when *F. temperatum* DNA was used (Figure 1f).

3.2. Study on the occurrence of mycotoxins and mycotoxin-producing fungi on maize samples

3.2.1. Detection of mycotoxigenic *Aspergillus* and *Fusarium* species by specific PCR assays

All the samples analyzed were positive for amplification using the primer set ITS1/ITS4, indicating the presence of fungal DNA in the samples and its suitability for PCR amplification. The results obtained on the occurrence of mycotoxigenic species, using species-specific PCR assays directly on maize samples at various stages of the production cycle are shown in Table 3. *Aspergillus ochraceus*, *A. westerdijkiae*, *A. steynii*, *F. subglutinans*, *F. temperatum*, *F. equiseti*, *F. culmorum*, *F. sporotrichioides*, *F. poae* and *F. langsethiae* were not detected in the samples using their specific PCR protocols and, therefore, these results are not included in the table.

It is important to note the presence of at least one mycotoxin-producing species in all the samples taken at the flowering stage. *Aspergillus flavus* was detected at this stage in all samples, at all seasons, and in each of the three batches. The presence of *A. niger* aggregate species (*A. niger* and *A. welwitschiae*) in flowering samples was also confirmed at all times. Contrastingly, the detection of *Fusarium* species was more variable, with three species being detected in 2018, and yet none in 2016.

In the pre-harvest samples, seven mycotoxin-producing species were detected (*F. verticillioides*, *F. proliferatum*, *A. flavus*, *A. parasiticus*, *A. carbonarius*, *A. niger* and *A. welwitschiae*); whereas, during storage in the silo, only five were found (*F. verticillioides*, *F. proliferatum*, *F. graminearum*, *A. flavus* and *A. welwitschiae*).

Regarding the presence of the different fungal species, *A. flavus* was the most frequently detected (89 % of samples), followed by the *A. niger* aggregate species (52 %). *Aspergillus parasiticus* were only found in a low percentage of pre-harvest samples (11 %). On the other hand, *A. carbonarius* was only detected in the flowering phase in 2017 and 2018.

Fusarium verticillioides and *F. proliferatum* were the only fumonisin-producing *Fusarium* species that were detected in the maize samples. *F. graminearum* was present in a low percentage of samples (15 %), and only in 2018.

Regarding the period of collection, a high number of different mycotoxin-producing species were detected at all crop stages in 2018 compared with the other seasons.

Table 3. PCR detection of *Fusarium* and *Aspergillus* species in pre-harvest and stored maize samples using species-specific assays. The presence (+) or absence (-) of the specific PCR amplification product is indicated for each replicate (1, 2, 3)

	F. verticillioides			*F. proliferatum*			*F. graminearum*			*A. flavus*			*A. parasiticus*			*A. carbonarius*			*A. niger*			*A. welwitschia*		
SAMPLES	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
P1.FM-16	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
P2.FM-16	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
P1.FF-16	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
P2.FF-16	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
P1.PRE-16	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+
P2.PRE-16	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	+	-	-	+	+	+
SILO-16	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
P1.FM-17	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-
P2.FM-17	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-
P3.FM-17	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
P1.FF-17	+	+	+	+	-	-	-	-	-	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-
P2.FF-17	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-
P3.FF-17	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+
P1.PRE-17	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
P2.PRE-17	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
P3.PRE-17	+	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	+	+	+	-	+
SILO-17	+	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
P2.FM-18	+	+	+	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+
P3.FM-18	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+
P4.FM-18	-	-	-	+	-	-	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+
P2.FF-18	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
P3.FF-18	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-
P4.FF-18	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	-	-	-	-	+	+	+
P2.PRE-18	+	+	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
P3.PRE-18	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P4.PRE-18	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
SILO-18	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+

PCR, polymerase chain reaction; (+) positive amplification and (-) no amplification. P (plot); MF (male flower); FF (female flower); PRE (pre-harvest).

3.2.2. Mycotoxin contamination

The results regarding mycotoxin analysis on the samples obtained at the moment of harvest or after three months of storage for three consecutive seasons are shown in Table 4. Deoxynivalenol (DON), type A trichothecenes (T-2 and HT-2), zearalenone (ZEA), aflatoxin B_1 (AFB_1), and ochratoxin A (OTA) were not detected in any of the samples at any stage.

Table 4. Fumonisin concentrations (FB_1 and FB_2) in the pre-harvest (PRE) and stored maize samples, across three seasons. Each value corresponds to the mean of two replicates ± standard error. The detection limits were 180 and 60 μg/kg in the cases of FB_1 and FB_2, respectively.

Season	Plot	Samples	FB_1 (μg/kg)	FB_2 (μg/kg)
2016	P1	PRE 1.1-16	1219.44 ± 2.21	214.86 ± 1.96
		PRE 1.2-16	1289.89 ± 214.75	218.15 ± 5.82
		PRE 1.3-16	204.14 ± 0.62	ND
	P2	PRE 2.1-16	ND	ND
		PRE 2.2-16	ND	ND
		PRE 2.3-16	1334.74 ± 31.57	168.71 ± 2.62
		SILO-16	ND	ND
		SILO-16	ND	ND
		SILO-16	ND	ND
2017	P1	PRE 1.1-17	ND	ND
		PRE 1.2-17	3658.78 ± 75.79	740.06 ± 4.08
		PRE 1.3-17	ND	ND
	P2	PRE 2.1-17	5902.87 ± 45.73	606.41 ± 29.06
		PRE 2.2-17	ND	ND
		PRE 2.3-17	1496.14 ± 33.05	312.32 ± 2.02
	P3	PRE 3.1-17	ND	ND
		PRE 3.2-17	ND	ND
		PRE 3.3-17	ND	ND
		SILO-17	ND	ND
		SILO-17	ND	ND
		SILO-17	ND	ND
2018	P2	PRE 2.1-18	371.53 ± 0.17	111.24 ± 3.24
		PRE 2.2-18	355.78 ± 6.83	85.86 ± 1.62
		PRE 2.3-18	ND	ND
	P3	PRE 3.1-18	870.63 ± 9.98	215.46 ± 5.22
		PRE 3.2-18	455.35 ± 6.30	111.78 ± 0.18
		PRE 3.3-18	ND	ND
	P4	PRE 4.1-18	ND	ND
		PRE 4.2-18	ND	ND
		PRE 4.3-18	ND	ND
		SILO-18	180.25 ± 1.40	ND
		SILO-18	198.98 ± 0.53	ND
		SILO-18	186.03 ± 0.88	ND

ND: non detected; P (plot); PRE (pre-harvest).

In the first season of sampling (2016), fumonisins type B (FB) were not detected in the silo, whereas the mean levels of the sum of fumonisins B_1 and B_2 (FB_1 and FB_2) in the two plots (P1 and P2) studied in pre-harvest were 1068.82 and 661.15 μg/kg, respectively. In no case did the values exceed the regulations for FBs in unprocessed corn, which are established at FB_1 and FB_2 > 4000 μg/kg [8].

At the second season (2017) FB_1 and FB_2 were not detected—neither in the stored grains nor the pre-harvest samples from plot P3. However, FBs were detected at higher levels than in 2016 in pre-harvest samples from the P1 and P2 plots. The FB levels even exceeded the limits established by the European Union in one of the batches from P2.

At the third season (2018), FBs were not detected in the pre-harvest samples from P4, whereas samples from P2 and P3 were contaminated by FBs at low values (mean values 388.04 and 631.07 μg/kg, respectively) in two out of the three batches.

During storage, FB_1 concentration values were near the detection limits of the analysis method and FB_2 was not detected in any of the silo samples.

4. Discussion

Maize is one of the most important food crops in the world [5]. However, its grains are susceptible to being contaminated by mycotoxin-producing fungi, both in the field and during storage, posing a serious risk to food safety [13]. Several authors have reported the occurrence of mycotoxigenic fungi in maize. *Fusarium verticillioides*, *F. proliferatum* and *F. graminearum* are considered the species that cause the most concern at pre-harvest [12,31], whereas *Aspergillus flavus* causes the most significant concern during the storage of maize [6]. Almost all information regarding the mycotoxigenic potential of these species and their ability to colonize maize comes from in vitro studies. However, the stress on developing maize—particularly during reproductive phases—facilitates infection by the fungi, mycotoxin production and the contamination of the grain [32]. Moreover, it has been reported that, in the field, fungal metabolism changes in order to adapt to unfavorable environmental conditions or limited nutritional availability [33,34]. These authors highlighted the relevance of studies similar to those presented in this manuscript. To date, to our knowledge, there are no studies on the occurrence of mycotoxins and mycotoxin producing-fungi along the complete maize production cycle. The present study is the first one that aims to include all the reproductive cycles and that is focused on the occurrence of the main mycotoxin-producing *Aspergillus* and *Fusarium* species in Spain, from the flowering of the crop to the storage of the grains.

One of the main objectives of this work was to determine the moment when the contamination with potential producers occurs and if it is related with the appearance of the corresponding mycotoxin. Mycotoxigenic fungi can infect maize, even causing ear rots, and afterwards can contaminate grains with mycotoxins [14]. The initial colonization, fungal development, and subsequent mycotoxin production may happen during the cultivation and/or storage of maize [12]. During the three years over which this study was conducted, some *Aspergillus* and *Fusarium* species were detected across the complete production cycle of flowering, harvest and storage. The presence of *A. flavus* was much more frequent and occurred at all stages; in comparison to the three *Fusarium* species detected, which were usually first detected at harvest. It is well-known that *A. flavus* and *F. verticillioides* usually co-occur in corn, since they are able to occupy different niches regarding carbon sources [34].

The only toxins detected in this study were fumonisins B_1 and B_2 (FB_1 and FB_2), the presence of which was consistent at pre-harvest and, in some seasons, reached high levels—although they did not exceed legal limits in silo. Corn is the most susceptible host to contamination, with FUM-producing species often in the field when grains present a high moisture content [35]. In our work, the highest levels of FUMs were observed in pre-harvest in 2017. That season, the weather at harvest time was humid and at high temperatures, with a rainfall of 50 L/m^2 and a maximum temperature of 42 °C [36], which might have effected an increase in grain moisture which would favor FUM production.

Several authors proposed different strategies to reduce fungal development as well as mycotoxin production in corn, such as the application of Good Agricultural Practices (GAP), chemical and biological control during cultivation, and proper management during harvest and storage [6,37]. In the farm where this study was carried out, the farmer applied GAP by avoiding stress due to drought or lack of nutrients, rotating crops, using early flowering corn-varieties, and allowing the plots to lie fallow after two or three years of corn cultivation. These measures—that are usually applied—surely should prevent the presence of mycotoxin levels that exceed European Regulations [8]. Removing crop residues, crop rotation, and the practice of fallow seasons are critical factors to prevent fungal colonization and the subsequent mycotoxin production of maize [6,37]. In our study, mycotoxins were not detected in the P3 plot in 2017, nor in P4 in 2018, and both plots lay fallow the preceding season. Furthermore, it is important to note that FBs were detected at the point of harvest in all the other plots that had not lay fallow the previous season—supporting the fact that GAP drastically affect the mycotoxin contamination of the grains.

In our study, mycotoxin levels in the silos were undetectable in the first two years of the study (2016 and 2017), whereas FB_1 and FB_2 were detected at very low levels in 2018. In all seasons, the concentrations found in stored samples were lower than those found at harvest. This might be due to the mixture of different fields in the silo and the grains not corresponding solely to the plots we selected for our study. Maize is one of the most susceptible products to mycotoxin contamination, due to deficient storage conditions, and the extremely low levels observed in this silo might be a reflection of the good agricultural and storage practices engaged in by the farmers. Water activity is one of the main factors affecting mycotoxin growth and production in maize by *A. flavus* and *F. verticillioides*, among others [38]; therefore, the application of this drying process makes the grains less susceptible to fungal colonization and mycotoxin production. In this field, after harvest, the cobs are threshed and homogenized, and the maize grains are subjected to a process of drying which clearly decreased the grain moisture up to levels of 7–8 %. These values are in agreement with FAO recommendations to maintain moisture levels less than 15 % to prevent fungal growth of fungal species that may be present on fresh grains [39]. Other GAPs that should be applied include the additional control of moisture and temperature during corn storage in silos is crucial to minimizing fungal growth and toxin development [40]. The facilities where this study was conducted were well-ventilated and adequate for the conservation of the corn grains. During the last season of the study, the humidity and temperature data of the silo were recorded across the three months of storage. The relative humidity values were below 65 % and the temperature values recorded were always below 10 °C, which are considered poor optimal values for mycotoxin production [41].

As mentioned before, detectable levels of FUMs were obtained in the maize grains after three months of storage, though only in 2018. In the same season, *F. graminearum* was also detected in the samples, co-occurring with FUM-producing species. Velluti et al. (2001) described how *F. graminearum* is able to stimulate the growth of *F. verticillioides* when co-occurring, increasing its ability to produce FB_1 [42]. Therefore, fungal interactions might be also relevant in assessing the mycotoxin risk in maize crops, which supports the importance of this kind of comprehensive study.

In a climate change scenario, aflatoxins (AFs) have been considered extremely significant as contaminants of maize, mainly in South European countries [13]. However, in our study, AFs were not detected in any samples, although *Aspergillus* section *Flavi* species were detected from the first stages of the production cycle. As mentioned before, AF-production by *Aspergillus* species is significantly affected by humidity and temperature [43]; consequently, maintaining appropriate storage conditions may reduce or even avoid AF contamination. Moreover, several recent studies have reported that many *A. flavus* isolates are unable to synthetize AFs, due to the presence of a non-functional biosynthetic cluster [44,45]. Therefore, it would be interesting to study the capacity of producing AFs of Spanish isolates to uncover the real risk posed by *A. flavus* on maize.

The *A. niger* aggregate species—*A. niger* and *A. welwitschiae*—were consistently detected across all three seasons and each of the steps of production cycle. However, ochratoxin A (OTA) was not found in any case. The characteristics of Spanish isolates of *A. niger* and *A. welwitschiae* have been recently studied and less than 15 % are OTA producers [46]. Most of them presented a truncated version of the OTA biosynthetic cluster, that resulted in the loss of their ability to produce the toxin. However, several authors have reported that *A. welwitschiae* and mainly *A. niger* are important FB_2 producers in maize [46,47]. Therefore, the presence of *A. niger* aggregate species discovered in the present work might be contributing to the FB_2 levels detected in corn samples.

The rapid and direct identification of the mycotoxigenic species that contaminate maize is a good indicator in predicting the risk of mycotoxin production in the grains during storage, offering an alternative to conventional microbiological procedures in fungal diagnostics. In this context, molecular techniques offer a good alternative tool to detect these species in maize samples [28]. In several published works by our group, species-specific PCR protocols have been developed and successfully applied to detect the main mycotoxin-producing species in cereals [48,49].

Previous studies have already optimized specific PCR assays to detect *Fusarium* species that are mycotoxin-producing or pathogens of maize [17,30]. However, the new taxonomic changes and/or the description of new *Fusarium* species of interest in relation to maize [50,51] have supposed that some of these previously described methods gave unspecific results. Therefore, in this work, we have developed and optimized new protocols to detect and identify *F. graminearum*, *F. langsethiae*, *F. fujikuroi*, *F. poae*, *F. sporotrichioides* and *F. temperatum*, using pure DNA or direct maize samples, and we also have modified the PCR conditions in the case of *F. subglutinans* to ensure the specific amplification using the primers described by Scauflaire et al. (2012) [30]. These assays have been tested using the purified DNAs of several isolates of different origins, as well as the DNAs of different, closely related *Fusarium* species frequently associated with corn. The application of these PCR assays directly onto corn grains allows the processing of a high number of samples and reduces the time of analysis compared to conventional methods [28]. In the corn samples analyzed in this work, we were only able to detect one of the species tested using these new optimized protocols—*F. graminearum*—in both flowers and corn grains. However, as mentioned before, the protocols were optimized using the pure DNAs of more than seventeen

Fusarium species, which guarantee that they may be successful in detecting these species if they are naturally occurring in maize samples.

5. Conclusion

Maize is one of the most susceptible cereals to contamination by mycotoxins and mycotoxigenic fungi. In this work, a comprehensive study across the maize production cycle was performed to discover when the contamination by mycotoxigenic fungi appears and its relation to mycotoxin presence in stored maize. The results indicated that *Aspergillus flavus* and fumonisin-producing *Fusarium* species are able to colonize at the earlier stages of the production cycle. However, the application of good agricultural and storage practices by farmers critically minimizes—or even avoids—mycotoxin contamination of maize grains.

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CHAPTER 2

Occurrence of *Fusarium* and *Aspergillus* species in maize and oat samples and their subproducts in Spain.

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Abstract

Maize and oat are consumed worldwide, being one of the main sources of food and feed. Their consumption in the last decade has drastically increased, since consumers look for healthy and nutritive products. However, cereal consumption might pose a risk for food safety because they can be contaminated by mycotoxins. Mycotoxin contamination occurs both in the field and during storage, which may result in high levels of this compounds occurring in cereal-based products. The objective of this study was to evaluate the situation of maize and oat cereals in Spain regarding the presence of potentially mycotoxigenic species. The detection of the main mycotoxin-producing species of *Fusarium* and *Aspergillus* genera was performed using species specific species PCR protocols and different types of samples were studied. On the one hand, samples of maize and oat were collected from the main cereal areas of Spain between 2016 and 2018, and, on the other hand, commercial samples of both maize and oat-products were obtained in the Spanish market. The most frequently detected toxigenic species were *Aspergillus flavus*, *A. niger*, *Fusarium verticillioides* and *F. proliferatum*. Subsequently, *Fusarium* and *Aspergillus* strains were isolated from cereal samples, and a phylogenetic analysis was performed to unravel whether intraspecific variability might be occurring. However, genetic relationships between the sources or location of isolation were not found in any case. The presence of potentially mycotoxigenic species in maize and oat samples might be carefully considered since it can be related to a probable mycotoxin contamination of these products.

Keywords

Mycotoxins; oat; maize; cereal-based products; PCR detection; phylogeny; *Aspergillus*; *Fusarium*.

1. Introduction

Maize (*Zea mays* L.) and oat (*Avena sativa* L.), together with wheat (*Triticum* spp.) and barley (*Hordeum vulgare* L.), are the most important cereals in the European Union, According to the Food and Agriculture Organization of the United Nations, the annual production of this region in 2018 was 138, 69, 46 and 7 million tons for wheat, maize, barley and oat, respectively. In Spain, an average of 6 million ha of cereals are cultivated every season. Barley (38.7 %), common wheat (28.9 %), maize (15.8 %), oat (6.2 %), durum wheat (2.9 %), triticale (1.7 %) and sorghum (0.1 %) are the main cereals cultivated in Spain [1].

In recent years, significant changes in human dietary habits were observed, including an increase in the use of products with higher nutritional content, such as cereals. Cereals are essential in human diet due to their high content of vitamins and minerals [2]. Consumers associate the consumption of cereal products such as breakfast oatmeal or energy bars with a healthy lifestyle because of their high fibre content [3]. The benefits of dietary fibre intake are numerous, including the improvement of intestinal function and carbohydrates and fat absorption, slowing digestion, and reduction of disease risk [4]. In addition, the increasing prevalence of allergies or food intolerances has led many companies to specialize in the production of gluten-free products, being oat and maize the main components [5].

Mycotoxins are fungal toxic secondary metabolites which present acute or chronic toxicity to animals and humans [6]. Cereals may be contaminated with toxigenic moulds, which can produce mycotoxins if the right conditions for their development occur, and these compounds are now considered one of the most important risks for food safety [7]. These potentially toxigenic fungi are able to grow in the cereal grains, and produce mycotoxins both in the field and during storage [8]. Early detection of mycotoxigenic fungi is crutial to prevent mycotoxins entering the food chain. These compounds are very stable and they are not degraded during most of technological processes used in food production. Therefore, the use of contaminated raw materials may suppose the occurrence of mycotoxins in the final products [9].

The most relevant mycotoxin-producing genera affecting maize are *Fusarium* and *Aspergillus* [10,11], whereas oat is mainly contaminated by *Fusarium* spp. [12,13]. The main mycotoxins associated with these cereals are fumonisins (FUM), trichothecenes (TCTs), zearalenone (ZEA), aflatoxins (AF), and ochratoxin A (OTA) [2,8]. Cereals and cereal-based foods can be contaminated by a single mycotoxin, although their co-occurrence is also common [4,14].

Due to their toxicological effects, the European Commission set the maximum allowed levels of some mycotoxins in 2006. These regulation includes the limits of AF, OTA, deoxynivalenol (DON (type B TCT)), and ZEA levels in all unprocessed cereals and cereal based products as well as AF, DON, ZEA and FUM in unprocessed maize and different maize-derived products for direct human consumption [15]. This regulation was subsequently modified in 2007, to apply more restrictions on the level of *Fusarium* toxins in maize and maize products [16]. In 2013, the European Commission published a recommendation on the presence of T-2 and HT-2 toxins in

cereals and cereal products [17]. However, currently, there is no legislation setting the maximum levels of T-2 and HT-2 toxins in cereals and based cereal products, and OTA in maize, due to a lack of studies regarding their relevance.

Mycotoxins can be detected long after the producing fungi have died out or been replaced by other species [18]. Several authors have reported that techniques based on PCR, which target the fungal DNA, are considered a good alternative for direct species-specific detection in food samples [19,20]. Moreover, the identification is rapid, since there is no need to perform a prior isolation of fungi. Species identification and detection are performed on the basis of genotypic differences, and these techniques are highly specific and sensitive, being able to detect target DNA molecules in complex mixtures even when the moulds are no longer viable [20,21].

Several works carried out in our laboratory have successfully developed specific PCR assays to detect mycotoxin-producing species in naturally contaminated cereals [11,22-28]. Currently, there is little information available on the occurrence of mycotoxin-producing species in oat crops in Spain. This kind of studies are necessary to provide baseline information and predict mycotoxin presence on products intended for food and feed in the European Union, in order to set limits and establish safer regulations.

The main objective of this study was to study the current situation of oat and maize crops, as well as their derived commercial-products distributed in Spain, regarding their contamination by toxigenic *Fusarium* and *Aspergillus* species. Species specific PCR protocols were applied to reveal the presence of mycotoxin-producing species in a variety of samples of these products. Additionally, a phylogenetic analysis was performed to study the possible intraspecific variability of the most frequently isolated species.

2. Materials and Methods

2.1. Cereal samples

Seventy-three oat samples (*Avena sativa* L.) and 45 maize samples (*Zea mays* L.) collected at harvest time have been analysed in this work (Tables 1 and 2, respectively). The samples came from the main cereal areas in Spain: Galicia, País Vasco (Guipúzcoa), Navarra, Aragón (Huesca and Zaragoza), Cataluña (Lleida), Castilla y León (León, Zamora, Salamanca, Palencia, Valladolid, Ávila, Burgos and Segovia), Madrid, Castilla la Mancha (Cuenca, Albacete, Ciudad Real and Toledo), Extremadura (Badajoz and Cáceres), Comunidad Valenciana (Valencia) and Andalucía (Sevilla). The samples were collected by the corresponding farmers at harvest time, at three consecutive seasons (2016, 2017 and 2018).

Additionaly, 25 commercial samples of different kind of cereal-based products distributed in Spanish markets (flours, breakfast cereals, sweet maize pasta, baby-food and snacks such as popcorn or fried corn) were also analysed in this study (Table 3).

All samples were milled with an IKA A11 Basic Mill (IKA, Königswinter, Germany) to obtain a fine powder, according to European Union requirements (CE 401/2006) [29]. The milled samples were stored at -20 °C in hermetic sterile plastic bags until analysis.

2.2. *PCR detection of the main mycotoxin-producing* **Fusarium** *and* **Aspergillus** *species in cereal samples*

2.2.1. DNA extraction

A short 24 hour-culture of the cereal samples were performed prior to DNA extraction to increase fungal biomass; therefore, 1 g of the corresponding milled sample was cultured in 50 mL of Sabouraud–Chloramphenicol broth (Pronadisa, Madrid, Spain) at 28 ± 1 °C on an orbital shaker SK-0330-PRO (140 rpm (Labolan, Navarra, Spain)) in darkness. Subsequently, the samples were filtered through Whatman Nº 1 paper. Filtered cultures were frozen with liquid nitrogen and grounded using a mortar and pestle. The samples were kept at - 80 °C until DNA extraction.

DNA extraction was carried out by triplicate using the NZYPlant/Fungi gDNA Isolation Kit (Nzytech, Lisbon, Portugal) according to manufacturer's instructions (DNA extraction protocol from fungi) starting from 100 mg of grounded sample. DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA).

2.2.2. Species-specific PCR assays

The specific PCR assays were performed in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). The amplification reactions were carried out in volumes of 25 μL containing 200 ng of total genomic DNA, 1 μL of each primer (20 μM; Metabion, Planegg, Germany) and 12.5 μL of NZYTaq II 2x Green Master Mix (Nzytech, Lisbon, Portugal). PCR products were detected in 2 % agarose ethidium bromide gels in TAE 1X buffer (Tris Acetate 40 mM and EDTA 1.0 mM). The NZYDNA Ladder V (Nzytech, Lisbon, Portugal) was used as molecular size marker.

Presence of fungal DNA was tested in all the samples using the universal primer set 5.8S1/5.8S2 [30]. Subsequently, species-specific PCR protocols were applied to detect the main mycotoxigenic species in cereals.

The presence of *Fusarium* species was tested using the protocols previously described in our laboratory for specific detection of *F. proliferatum* [25], *F. verticillioides* [22], *F. equiseti, F. culmorum* [24], *F. subglutinans, F. temperatum, F. graminearum, F. poae, F. sporotrichioides,* and *F. langsethiae* [11]. The detection of the main mycotoxigenic *Aspergillus* species was also performed using species-specific PCR protocols in the case of *A. flavus* [26], *A. parasiticus* [28], *A. carbonarius* [23], *A. westerdijkiae, A. ochraceus*, and *A. steynii*, [27] as well as *A. niger* and *A. welwitschiae* [31]. All DNA samples were analysed in triplicate.

2.3. *Phylogenetical analysis of the main isolated* **Fusarium** *and* **Aspergillus** *species in cereal samples*

2.3.1. Fungal isolates and culture conditions

One gram of each milled cereal sample was homogenized with 9 ml of sterile saline solution (0.9 g/L sodium chloride (Merck, Darmstadt, Germany) and serially diluted. Subsequently, a volume of 100 µl of each dilution was spread (in triplicate) on the surface of Petri plates containing Rose Bengal-Chloramphenicol Agar (Pronadisa, Spain). The plates were incubated at 28 °C in the dark for 2 days, and then for 3 days under 12 h light and dark cycles.

All colonies morphologically assigned to *Fusarium* and *Aspergillus* genera, were re-isolated on potato dextrose agar medium (PDA, Pronadisa, Madrid, Spain) at 25 ± 1 °C for 5 days and stored as spore suspensions in 15 % glycerol (Panreac, Madrid, Spain) at -80 °C until required. For the taxonomic identification of fungi, the criteria of Barnett and Hunter [32] were followed.

2.3.2. DNA extraction and sequencing

Genomic DNA extraction from *Fusarium* and *Aspergillus* strains isolated from cereal samples was carried out using the protocol described by Querol [33]. Fungal mycelia from 4-day-old cultures on PDA agar plates were scraped off with a scalpel, frozen with liquid nitrogen and grinded using a micropistille before DNA extraction. DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA).

The amplification reactions using 100 ng of pure genomic DNA and the detection of the PCR products were carried out as described in the section 2.2.2 (Species-specific PCR assays). In order to perform phylogenetic studies, a partial sequence of the β-tubulin encoding gene was amplified in the case of section *Flavi* isolates using the protocol described by Glass and Donaldson [34], whereas the partial sequence of the elongation factor encoding gene gene (*ef-1α*) was amplified in *Fusarium* strains using the protocol described by O´Donnell [35]. A partial fragment of the calmodulin encoding gene was amplified in all *A. niger* aggregate isolates using the PCR primers described by Peterson [36], and the following conditions: an initial denaturalization cycle of 5 min at 95 °C, 36 cycles of 30 s at 95 °C, 30 s at 54 °C and 50 s at 72 °C, and a final extension cycle of 7 min at 72 °C.

The amplification products of approximately 450, 600 and 670 bp long for β-tubulin, ef-1*α* and calmodulin encoding genes, respectively, were purified using the NZYGelpure kit (Nzytech, Lisboa, Portugal). Sequencing was performed in an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Foster City, USA) according to manufacturer's instructions in Macrogen facilities (Madrid, Spain). All amplification products were sequenced in both directions. Sequences were assembled using the UGENE 1.29 package (Unipro, Novosibirsk, Russia). The sequences were compared with those deposited on the NCBI nucleotide database to reach the identification of the corresponding isolates at species level.

Subsequently, sequences were aligned using the algorithm MUSCLE [37], and a phylogeny was inferred using the Neighbor-Joining method [38] with 1,000 bootstrap replicates. Phylogenetic and molecular evolutionary analyses were conducted using MEGA X [39].

3. Results

3.1. PCR detection of the main mycotoxin-producing Fusarium *and* Aspergillus *species in cereal samples*

A band of the expected size was obtained using DNA from all samples analysed using the primer set 5.8S1/5.8S2, indicating the presence of fungal DNA and, therefore, the species-specific PCR protocols were performed using all the samples. The results obtained are indicated in Table 1 for oat samples, Table 2 for maize samples and Table 3 for commercial samples. These tables only include the results regarding PCR assays in which at least one positive sample was detected.

Table 1. Oat samples analyzed indicating harvest season and location as well as the presence (+) or absence (-) of the species-specific PCR amplification product of *F. verticillioides, F. equiseti, F. graminearum, A. flavus, A. parasiticus, A. carbonarius, A. niger, A. welwitschiae* and *A. westerdijkiae.*

DATE	LOCATION		*F. verticillioides*	*F. equiseti*	*F. graminearum*	*A. flavus*	*A. parasiticus*	*A. carbonarius*	*A.niger*	*A. welwitschiae*	*A. westerdijkiae*
2016	ANDALUCIA	SEVILLA	-	-	-	-	-	-	-	-	-
2016	ARAGÓN	-	-	-	-	+	-	-	-	-	-
2016	COMUNIDAD VALENCIA	VALENCIA	-	-	-	+	-	-	-	-	-
2016	CASTILLA LA MANCHA	CIUDAD REAL	-	-	-	+	-	-	+	-	-
2016	CASTILLA LA MANCHA	CIUDAD REAL	-	-	-	+	-	-	-	-	-
2016	CASTILLA LA MANCHA	CIUDAD REAL	-	-	-	+	-	-	+	-	-
2016	CASTILLA LA MANCHA	CUENCA	-	-	-	-	-	-	-	-	-
2016	CASTILLA LA MANCHA	CUENCA	-	-	-	-	-	-	-	-	-
2016	CASTILLA LA MANCHA	CUENCA	-	-	-	+	-	-	-	-	-
2016	CASTILLA LA MANCHA	CUENCA	-	-	-	+	-	-	-	-	-
2016	CASTILLA LA MANCHA	CUENCA	-	-	-	-	-	-	-	-	-
2016	CASTILLA LA MANCHA	CUENCA	-	-	-	-	-	-	-	-	-
2016	CASTILLA LA MANCHA	CUENCA	-	-	-	+	+	-	-	-	-
2016	CASTILLA LA MANCHA	CUENCA	-	-	-	+	-	-	+	-	-
2016	CASTILLA Y LEÓN	BURGOS	-	-	-	-	-	-	-	-	-
2016	CASTILLA Y LEÓN	BURGOS	-	-	-	+	-	-	-	-	-
2016	CASTILLA Y LEÓN	BURGOS	-	-	-	-	-	-	-	-	-
2016	CASTILLA Y LEÓN	BURGOS	-	-	-	+	-	-	-	-	-
2016	CASTILLA Y LEÓN	LEÓN	-	-	-	+	-	-	-	-	-
2016	CASTILLA Y LEÓN	LEÓN	-	-	-	+	-	-	-	-	-
2016	CASTILLA Y LEÓN	LEÓN	-	-	-	-	-	-	-	-	-
2016	CASTILLA Y LEÓN	LEÓN	-	-	-	+	-	-	-	-	-
2016	CASTILLA Y LEÓN	SALAMANCA	-	-	-	+	-	-	-	-	-
2016	CASTILLA Y LEÓN	SALAMANCA	-	-	-	+	-	-	-	-	-

Cont. Table 1

2016	CASTILLA Y LEÓN	VALLADOLID	-	-	-	-	-	+	-	-	-
2016	CASTILLA Y LEÓN	VALLADOLID	-	-	-	+	-	-	-	-	-
2016	CASTILLA Y LEÓN	VALLADOLID	-	-	-	-	-	-	+	-	-
2016	CASTILLA Y LEÓN	VALLADOLID	-	-	-	-	-	-	+	-	-
2016	CASTILLA Y LEÓN	ZAMORA	-	-	-	-	-	-	-	-	-
2016	NAVARRA	-	-	-	-	+	-	-	-	+	-
2016	NAVARRA	-	-	-	-	-	-	-	-	-	-
2016	NAVARRA	-	-	-	-	+	-	-	-	-	-
2017	ANDALUCIA	SEVILLA	-	+	-	+	+	-	+	+	-
2017	ANDALUCIA	SEVILLA	-	-	-	+	-	-	-	-	-
2017	ANDALUCIA	SEVILLA	-	-	+	+	-	-	+	+	-
2017	ANDALUCIA	SEVILLA	-	-	-	+	-	-	-	-	-
2017	MADRID	-	-	-	-	+	-	-	+	-	-
2017	MADRID	-	-	-	-	+	-	-	-	-	-
2017	CASTILLA LA MANCHA	ALBACETE	-	-	-	+	-	-	-	-	-
2017	CASTILLA LA MANCHA	CIUDAD REAL	-	-	-	+	-	-	-	-	-
2017	CASTILLA LA MANCHA	CIUDAD REAL	-	-	-	+	-	-	-	-	-
2017	CASTILLA LA MANCHA	TOLEDO	+	-	-	+	-	-	+	+	-
2017	CASTILLA Y LEÓN	AVILA	+	-	-	+	-	-	+	-	-
2017	CASTILLA Y LEÓN	BURGOS	-	+	-	+	-	-	+	-	-
2017	CASTILLA Y LEÓN	BURGOS	-	-	-	+	-	-	-	-	-
2017	CASTILLA Y LEÓN	BURGOS	-	-	-	+	-	-	-	-	-
2017	CASTILLA Y LEÓN	VALLADOLID	-	-	-	+	+	-	-	-	-
2017	CASTILLA Y LEÓN	VALLADOLID	-	-	-	+	-	-	-	-	-
2017	NAVARRA	-	-	-	-	-	-	-	-	-	-
2018	ANDALUCIA	SEVILLA	-	-	-	-	-	-	-	-	-
2018	ARAGÓN	-	-	-	-	+	-	-	-	-	-
2018	MADRID	-	+	-	-	-	-	-	-	-	-
2018	CASTILLA LA MANCHA	ALBACETE	-	-	-	-	-	-	-	-	-
2018	CASTILLA LA MANCHA	CIUDAD REAL	-	-	-	+	-	-	-	-	-
2018	CASTILLA LA MANCHA	CUENCA	-	+	-	+	-	-	-	-	-
2018	CASTILLA LA MANCHA	CUENCA	-	-	-	+	-	-	+	-	-
2018	CASTILLA LA MANCHA	CUENCA	-	-	-	+	-	-	-	+	-
2018	CASTILLA LA MANCHA	CUENCA	-	+	-	+	-	-	+	-	-
2018	CASTILLA LA MANCHA	CUENCA	-	+	-	+	-	-	-	-	-
2018	CASTILLA LA MANCHA	CUENCA	-	-	-	-	-	-	-	-	-
2018	CASTILLA Y LEÓN	LEÓN	-	-	-	-	-	-	-	-	-
2018	CASTILLA Y LEÓN	BURGOS	-	-	-	-	-	-	-	-	+
2018	CASTILLA Y LEÓN	PALENCIA	-	-	-	-	-	-	-	-	-
2018	CASTILLA Y LEÓN	SALAMANCA	+	-	-	-	-	-	-	-	-
2018	CASTILLA Y LEÓN	SALAMANCA	-	-	-	-	-	-	-	-	-
2018	CASTILLA Y LEÓN	VALLADOLID	-	-	-	-	-	-	-	-	-
2018	CASTILLA Y LEÓN	VALLADOLID	-	-	-	-	-	-	-	-	-
2018	CASTILLA Y LEÓN	VALLADOLID	+	-	-	-	-	-	-	-	-
2018	CASTILLA Y LEÓN	VALLADOLID	-	-	-	+	-	-	-	-	-
2018	CASTILLA Y LEÓN	VALLADOLID	-	-	+	+	-	-	-	-	-
2018	CASTILLA Y LEÓN	VALLADOLID	-	-	-	+	-	-	-	-	-
2018	EXTREMADURA	-	-	-	-	+	-	-	-	-	-
2018	NAVARRA	-	-	-	-	-	-	-	-	-	-

Out of the 73 oat samples tested, 77 % were positive for at least one potentially toxigenic species. *Aspergillus flavus* was the most frequently detected species (63 % of positive samples) followed by *A. niger* (18 %), and *A. welwitschiae* which was detected in 7 % of the oat samples. Regarding *Fusarium* species only *F. verticillioides*, *F. equiseti* and *F. graminearum* were detected although in a low percentage (< 7 %).

In no case *F. proliferatum F. subglutinans, F. temperatum, F. culmorum, F. sporotrichioides, F. poae, F. langsethiae, A. ochraceus* nor *A. steynii* were detected in the oat samples analysed.

Table 2. Maize samples analyzed indicating harvest season and location as well as the presence (+) or absence (-) of the species-specific PCR amplification product of *F. verticillioides, F. proliferatum, F. subglutinans, F. temperatum, F. graminearum, A. flavus, A. parasiticus, A. niger, A. welwitschiae,* and *A. ochraceus.*

DATE	LOCATION		*F. verticillioides*	*F. proliferatum*	*F. subglutinans*	*F. temperatum*	*F. graminearum*	*A. flavus*	*A. parasiticus*	*A.niger*	*A. welwitschiae*	*A. ochraceus*
2016	ARAGÓN	-	+	+	-	-	+	-	-	-	-	-
2016	ARAGÓN	-	-	-	-	-	-	+	-	-	-	-
2016	ARAGÓN	-	+	+	-	-	-	+	-	-	-	-
2016	ARAGÓN	HUESCA	-	-	-	-	-	-	-	-	-	-
2016	MADRID	-	+	+	-	-	-	+	-	-	-	-
2016	COMUNIDAD VALENCIA	VALENCIA	+	+	-	+	-	+	-	-	-	+
2016	COMUNIDAD VALENCIA	VALENCIA	-	-	-	-	-	-	-	-	-	-
2016	COMUNIDAD VALENCIA	VALENCIA	-	-	-	-	-	+	-	-	-	-
2016	CASTILLA LA MANCHA	ALBACETE	+	+	-	-	+	-	-	-	-	-
2016	CASTILLA Y LEÓN	LEON	-	+	-	-	-	+	-	-	-	-
2016	CASTILLA Y LEÓN	LEON	-	-	-	-	-	+	-	-	-	-
2016	CASTILLA Y LEÓN	VALLADOLID	-	-	-	-	-	+	-	-	-	-
2016	CASTILLA Y LEÓN	VALLADOLID	-	+	+	-	-	-	-	-	-	-
2016	EXTREMADURA	BADAJOZ	+	+	-	-	-	-	-	-	-	-
2016	EXTREMADURA	BADAJOZ	-	-	-	-	+	-	-	-	-	-
2016	EXTREMADURA	CÁCERES	-	-	-	-	+	+	-	-	-	-
2016	GALICIA	-	-	-	-	-	+	-	-	-	-	-
2016	GALICIA	-	-	-	-	-	+	+	-	-	-	-
2016	GALICIA	-	-	-	-	-	+	-	-	-	-	-
2016	GALICIA	-	+	+	-	-	+	-	-	-	-	-
2017	ARAGÓN	-	+	+	-	-	-	+	-	-	+	-
2017	ARAGÓN	HUESCA	+	+	-	-	-	+	-	-	-	-
2017	ARAGÓN	ZARAGOZA	+	-	-	-	-	+	-	-	+	-
2017	ARAGÓN	ZARAGOZA	+	+	-	-	-	+	-	+	+	-
2017	MADRID	-	+	-	-	-	-	+	-	-	-	-
2017	MADRID	-	+	+	-	-	-	+	-	-	-	-
2017	COMUNIDAD VALENCIA	VALENCIA	+	-	-	-	-	+	-	-	-	-
2017	CASTILLA LA MANCHA	ALBACETE	+	+	-	-	-	+	-	-	-	-
2017	CASTILLA LA MANCHA	ALBACETE	-	-	-	-	-	+	-	-	-	-
2017	CASTILLA Y LEÓN	SEGOVIA	+	+	-	-	-	+	+	+	-	-
2017	CATALUÑA	LLEIDA	+	+	-	-	-	+	-	+	+	-
2017	GALICIA	-	+	-	-	-	-	+	-	-	-	-
2017	PAÍS VASCO	GUIPUZCOA	+	-	-	-	-	+	-	+	+	-
2018	ARAGÓN	-	+	+	-	-	-	+	-	-	-	-
2018	ARAGÓN	HUESCA	+	-	-	-	-	+	-	-	-	-
2018	ARAGÓN	HUESCA	+	-	-	+	+	+	-	-	-	-
2018	MADRID	MADRID	+	+	-	-	+	+	-	-	+	-
2018	CASTILLA Y LEÓN	LEON	+	+	-	-	+	+	-	-	-	-
2018	CASTILLA Y LEÓN	SEGOVIA	-	-	-	-	-	+	-	-	-	-
2018	CATALUÑA	LLEIDA	+	-	-	-	-	+	-	-	-	-
2018	EXTREMADURA	CACERES	+	-	-	-	-	+	-	-	-	-

Cont. Table 2

2018	EXTREMADURA	CACERES	+	-	-	-	-	+	+	-	-	-
2018	EXTREMADURA	CÁCERES	-	+	-	+	+	+	-	-	-	-
2018	GALICIA	-	-	-	-	+	+	-	-	-	-	-
2018	PAÍS VASCO	GUIPUZCOA	+	+	-	-	-	+	-	-	-	-

A high percentage of maize samples (98 %) were positive for at least one potentially toxigenic species, and more than 50 % of the samples were contaminated by 3 or more species. *Aspergillus flavus*, *F. verticillioides*, *F. proliferatum* and *F. graminearum* were the most frequently detected species, with percentages of 76, 62, 47 and 29 %, respectively. *Fusarium temperatum* (9 %) and the mycotoxigenic species of the *A. niger* aggregate (*A. niger* and *A. welwitschiae*) were also frequently detected (22 % of positive samples).

In no case *F. equiseti*, *F. culmorum*, *F. sporotrichioides*, *F. poae* and *F. langsethiae*, *A. carbonarius*, *A. westerdijkiae* and *A. steynii* were detected in the maize samples analysed.

Table 3. Commercial cereal-based products marketed in Spain analysed in this work. The type of product and presence (+) or absence (-) of the specific PCR amplification product of *F. verticillioides, F. proliferatum, F. poae, A. flavus, A. niger* and *A. welwitschiae* are indicated.

TYPE OF PRODUCT		*F. verticillioides*	*F. proliferatum*	*F. poae*	*A. flavus*	*A.niger*	*A. welwitschiae*
MAIZE	FLAKES (ORGANIC)	-	-	-	-	-	-
MAIZE	FLAKES (ORGANIC)	-	-	-	-	-	-
MAIZE	FLOUR	+	+	-	-	-	-
MAIZE	FLOUR	+	+	-	-	-	-
MAIZE	FRIED MAIZE	+	+	-	-	+	-
MAIZE	FRIED MAIZE	+	-	-	-	-	-
MAIZE	PASTA	+	+	-	-	-	-
MAIZE	POPCORN	+	+	-	-	-	-
MAIZE	POPCORN	-	-	-	-	-	-
MAIZE	POPCORN	+	-	-	-	+	+
MAIZE	POPCORN (ORGANIC)	-	-	-	+	-	-
MAIZE	SEMOLINA	+	+	-	-	+	-
MAIZE	SWEET CORN	+	+	-	-	-	-
MAIZE/OAT	BABY FOOD	+	+	-	-	-	-
OAT	BRAN (ORGANIC)	-	-	-	-	-	-
OAT	FLAKES	-	-	-	-	-	-
OAT	FLAKES	-	-	-	-	-	-
OAT	FLAKES	-	-	-	-	-	-
OAT	FLAKES (ORGANIC)	-	-	-	-	-	-
OAT	FLAKES (ORGANIC)	-	-	+	-	-	-
OAT	FLAKES (ORGANIC)	-	-	-	-	-	-
OAT	FLAKES (ORGANIC)	-	-	-	-	-	-
OAT	FLAKES (ORGANIC)	-	-	-	+	-	-

Cont. Table 3

OAT	FLOUR	-	-	-	+	-	-
OAT	FLOUR	+	-	-	+	-	+

Sixty per cent of commercial samples analysed were positive for at least one potentially toxigenic species. Maize-based samples (79 %) showed higher contamination than the oat-based samples (45 %). *Fusarium verticillioides, F. proliferatum, A. flavus* and *A. niger* were detected with in 44, 32, 16 and 12 % of the samples, respectively. The rest of the 12 species analysed were not detected in any commercial sample.

3.2. Phylogenetic analysis of the main **Fusarium** *and* **Aspergillus** *species detected in cereal samples*

Fifty *Fusarium* and 66 *Aspergillus* strains were isolated of cereal samples (Table 4). They were identified as *F. verticillioides* (32), *F. proliferatum* (16), *F. langsethiae* (1), *F. graminearum* (1), A. *tubingensis* (19), *A. niger* (4), *A. welwitschiae* (5), *A. flavus* (37) and *A. oryzae* (1).

Table 4. Fungal strains analyzed in the phylogenetic studies, indicating the source of isolation and the location as well as the species they belong to.

CODE	SOURCE	LOCATION		SPECIES
FLA MAI-A15	MAIZE COMMERCIAL	-		*A. flavus*
FLA MAI-A16	MAIZE COMMERCIAL	-		*A. flavus*
FLA MAI-A32	MAIZE COMMERCIAL	-		*A. flavus*
FLA MAI-A43	MAIZE COMMERCIAL	-		*A. flavus*
FLA MAI-A44	MAIZE COMMERCIAL	-		*A. flavus*
FLA MAI-A45	MAIZE COMMERCIAL	-		*A. flavus*
FLA MAI-A46	MAIZE COMMERCIAL	-		*A. flavus*
FLA MAI-A47	MAIZE COMMERCIAL	-		*A. flavus*
FLA MAI-A28	MAIZE HARVEST	ARAGÓN	-	*A. flavus*
FLA MAI-A29	MAIZE HARVEST	ARAGÓN	-	*A. flavus*
FLA MAI-A38	MAIZE HARVEST	ARAGÓN	-	*A. flavus*
FLA MAI-A39	MAIZE HARVEST	ARAGÓN	-	*A. flavus*
FLA MAI-A40	MAIZE HARVEST	ARAGÓN	-	*A. flavus*
FLA MAI-A41	MAIZE HARVEST	ARAGÓN	-	*A. flavus*
FLA MAI-A2	MAIZE HARVEST	C.MADRID	-	*A. flavus*
FLA MAI-A3	MAIZE HARVEST	C.MADRID	-	*A. flavus*
FLA MAI-A4	MAIZE HARVEST	C.MADRID	-	*A. flavus*
FLA MAI-A7	MAIZE HARVEST	C.MADRID	-	*A. flavus*
FLA MAI-A11	MAIZE HARVEST	C.MADRID	-	*A. flavus*
FLA MAI-A13	MAIZE HARVEST	C.MADRID	-	*A. flavus*
FLA MAI-A14	MAIZE HARVEST	C.MADRID	-	*A. flavus*
FLA MAI-A24	MAIZE HARVEST	C.MADRID	-	*A. flavus*
FLA MAI-A36	MAIZE HARVEST	C.MADRID	-	*A. flavus*
FLA MAI-A37	MAIZE HARVEST	C.MADRID	-	*A. flavus*
FLA MAI-A55	MAIZE HARVEST	C.MADRID	-	*A. flavus*
FLA MAI-A56	MAIZE HARVEST	C.MADRID	-	*A. flavus*
FLA MAI-A25	MAIZE HARVEST	CASTILLA Y LEÓN	LEÓN	*A. flavus*
FLA MAI-A26	MAIZE HARVEST	CASTILLA Y LEÓN	LEÓN	*A. flavus*

Cont. Table 4

FLA MAI-A33	MAIZE	HARVEST	EXTREMADURA	BADAJOZ	*A. flavus*
FLA OAT-A10	OAT	COMMERCIAL	-		*A. flavus*
FLA OAT-A17	OAT	COMMERCIAL	-		*A. flavus*
FLA OAT-A18	OAT	COMMERCIAL	-		*A. flavus*
FLA OAT-A1	OAT	HARVEST	C.MADRID	-	*A. flavus*
FLA OAT-A50	OAT	HARVEST	C.MADRID	-	*A. flavus*
FLA OAT-A30	OAT	HARVEST	CASTILLA LA MANCHA	TOLEDO	*A. flavus*
FLA OAT-A51	OAT	HARVEST	CASTILLA LA MANCHA	CUENCA	*A. flavus*
FLA OAT-A9	OAT	HARVEST	CASTILLA Y LEÓN	VALLADOLID	*A. flavus*
NIG MAI-AN25	MAIZE	HARVEST	C.MADRID	-	*A. niger*
NIG OAT-A23	OAT	HARVEST	ANDALUCIA	SEVILLA	*A. niger*
NIG OAT-AN12	OAT	HARVEST	C.MADRID	-	*A. niger*
NIG OAT-AN11	OAT	HARVEST	CASTILL LA MANCHA	TOLEDO	*A. niger*
NIG OAT-AN13	OAT	HARVEST	CASTILLA Y LEÓN	VALLADOLID	*A. niger*
ORY OAT-A42	OAT	HARVEST	CASTILLA Y LEÓN	BURGOS	*A. oryzae*
TUB MAI-AN21	MAIZE	COMMERCIAL	-		*A. tubingensis*
TUB MAI-AN14	MAIZE	HARVEST	C.MADRID	-	*A. tubingensis*
TUB MAI-AN15	MAIZE	HARVEST	C.MADRID	-	*A. tubingensis*
TUB MAI-AN16	MAIZE	HARVEST	C.MADRID	-	*A. tubingensis*
TUB MAI-AN17	MAIZE	HARVEST	C.MADRID	-	*A. tubingensis*
TUB MAI-AN18	MAIZE	HARVEST	C.MADRID	-	*A. tubingensis*
TUB MAI-AN19	MAIZE	HARVEST	C.MADRID	-	*A. tubingensis*
TUB MAI-AN20	MAIZE	HARVEST	C.MADRID	-	*A. tubingensis*
TUB MAI-AN23	MAIZE	HARVEST	C.MADRID	-	*A. tubingensis*
TUB MAI-AN24	MAIZE	HARVEST	C.MADRID	-	*A. tubingensis*
TUB MAI-AN22	MAIZE	HARVEST	CASTILLA Y LEÓN	LEÓN	*A. tubingensis*
TUB OAT-AN5	OAT	HARVEST	CASTILLA LA MANCHA	CUENCA	*A. tubingensis*
TUB OAT-AN1	OAT	HARVEST	CASTILLA Y LEÓN	LEÓN	*A. tubingensis*
TUB OAT-AN2	OAT	HARVEST	CASTILLA Y LEÓN	LEÓN	*A. tubingensis*
TUB OAT-AN3	OAT	HARVEST	CASTILLA Y LEÓN	VALLADOLID	*A. tubingensis*
TUB OAT-AN4	OAT	HARVEST	CASTILLA Y LEÓN	VALLADOLID	*A. tubingensis*
TUB OAT-AN7	OAT	HARVEST	CASTILLA Y LEÓN	BURGOS	*A. tubingensis*
TUB OAT-AN8	OAT	HARVEST	CASTILLA Y LEÓN	BURGOS	*A. tubingensis*
TUB OAT-AN9	OAT	HARVEST	CASTILLA Y LEÓN	BURGOS	*A. tubingensis*
WEL MAI-AN29	MAIZE	COMMERCIAL	-		*A. welwitschiae*
WEL MAI-AN31	MAIZE	HARVEST	ARAGÓN	-	*A. welwitschiae*
WEL MAI-AN27	MAIZE	HARVEST	C.MADRID	-	*A. welwitschiae*
WEL MAI-AN37	MAIZE	HARVEST	C.MADRID	-	*A. welwitschiae*
WEL OAT-AN10	OAT	HARVEST	CASTILLA LA MANCHA	CUENCA	*A. welwitschiae*
GRAM MAI-F61	MAIZE	HARVEST	EXTREMADURA	BADAJOZ	*F. graminearum*
LAN OAT-F23	OAT	HARVEST	CASTILLA Y LEÓN	VALLADOLID	*F. langsethiae*
PRO MAI-F47	MAIZE	COMMERCIAL	-		*F. proliferatum*
PRO MAI-F69	MAIZE	HARVEST	ARAGON	-	*F. proliferatum*
PRO MAI-F2	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*
PRO MAI-F4	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*
PRO MAI-F5	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*
PRO MAI-F6	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*
PRO MAI-F8	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*
PRO MAI-F10	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*
PRO MAI-F11	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*
PRO MAI-F21	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*
PRO MAI-F22	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*
PRO MAI-F38	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*
PRO MAI-F40	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*
PRO MAI-F58	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*
PRO MAI-F66	MAIZE	HARVEST	C.MADRID	-	*F. proliferatum*

Cont. Table 4

PRO MAI-F71	MAIZE	HARVEST	GALICIA	-	*F. proliferatum*
VER MAI-F43	MAIZE	COMMERCIAL	-		*F. verticillioides*
VER MAI-F46	MAIZE	COMMERCIAL	-		*F. verticillioides*
VER MAI-F67	MAIZE	COMMERCIAL	-		*F. verticillioides*
VER MAI-F1	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F3	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F9	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F13	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F14	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F15	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F16	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F17	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F18	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F19	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F20	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F31	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F36	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F37	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F41	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F54	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F55	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F56	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F64	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F73	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F74	MAIZE	HARVEST	C.MADRID	-	*F. verticillioides*
VER MAI-F60	MAIZE	HARVEST	CASTILLA LA MANCHA	ALBACETE	*F. verticillioides*
VER MAI-F70	MAIZE	HARVEST	CASTILLA Y LEÓN	LEON	*F. verticillioides*
VER MAI-F53	MAIZE	HARVEST	EXTREMADURA	CACERES	*F. verticillioides*
VER MAI-F59	MAIZE	HARVEST	EXTREMADURA	BADAJOZ	*F. verticillioides*
VER MAI-F65	MAIZE	HARVEST	EXTREMADURA	CACERES	*F. verticillioides*
VER MAI-F72	MAIZE	HARVEST	EXTREMADURA	CACERES	*F. verticillioides*
VER OAT-F63	OAT	HARVEST	ARAGÓN	-	*F. verticillioides*
VER OAT-F48	OAT	HARVEST	CASTILLA Y LEÓN	BURGOS	*F. verticillioides*

The phylogenetic analysis performed on the basis of the partial sequence of the *ef-1α* gene of the 48 *Fusarium* isolates from this study. Most of *Fusarium* isolates from maize and oat were classified as *F. proliferatum* and *F. verticillioides*. One *F. langsethiae* and one *F. graminearum* isolate were also obtained in this work, and both were used as outgroup taxa for phylogenetical analysis. The Neighbour-Joining based tree obtained is shown in Figure 1. The evolutionary distances were computed using the Kimura 2-parameter method [40] and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.35). The dendrogram clearly clustered all *F. proliferatum* and *F. verticilliodes* isolates into two different branches supported by high bootstrap values. There was no apparent relationship between the classification of the isolates and their isolation source.

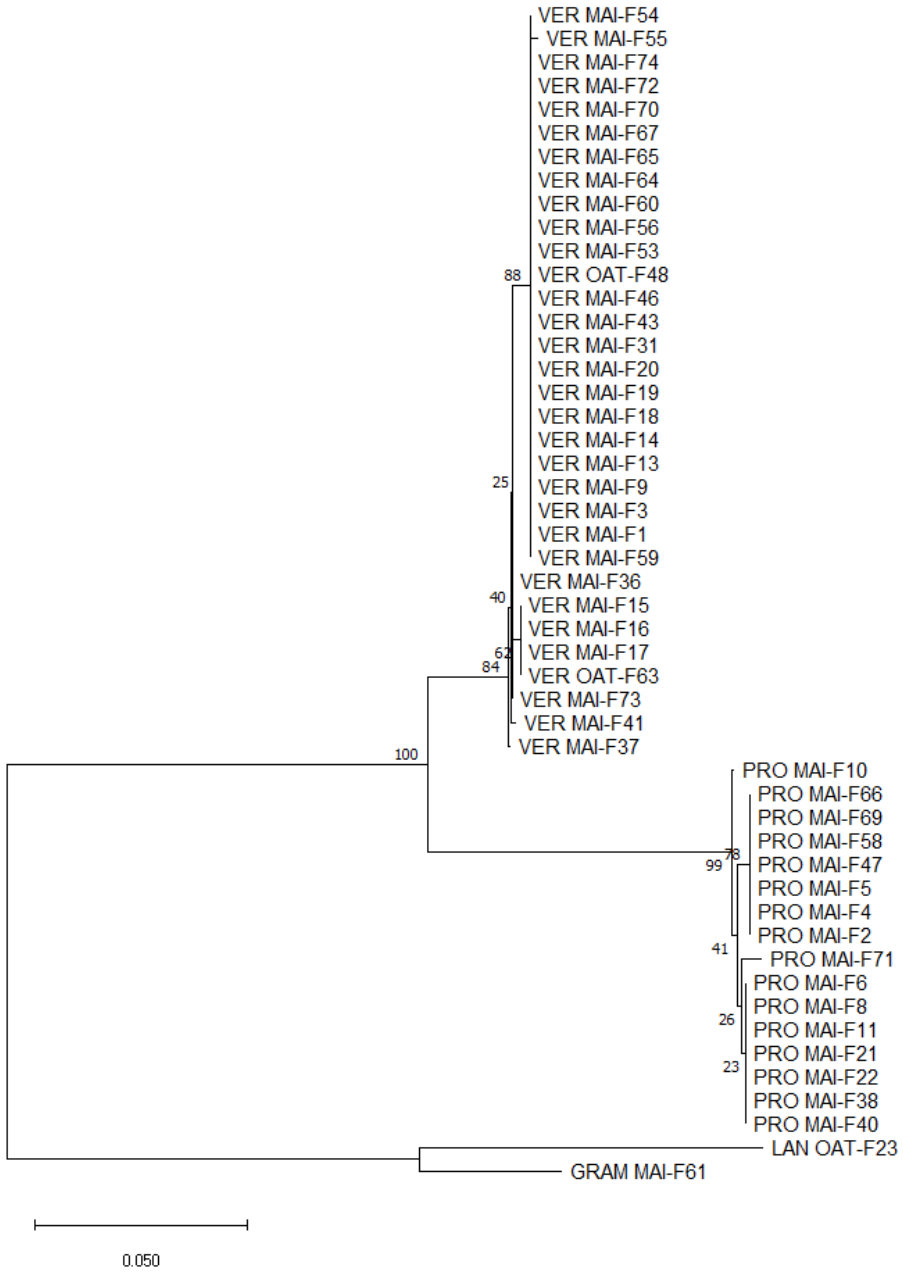

Figure 1. Phylogenetic analysis for 48 *Fusarium proliferatum* and *F. verticillioides* isolates from oat and maize, based on the sequence of a partial region of the *ef-1α* gene (504 nucleotide positions). The tree was constructed by the Neighbour-Joining method with 1,000 bootstrap replicates, using the Kimura 2-parameter method following a gamma distribution (shape parameter 0.35). The optimal tree with the sum of branch length = 0.42400669 is shown.

A similar phylogenetic analysis was performed using the partial sequences of the calmodulin encoding gene of 28 *A. niger* aggregate isolates obtained in this work (*A. niger, A. tubingensis* and *A. welwitschiae*), and the tree is shown in Figure 2. The corresponding sequence of one *A. flavus* isolate from maize was used as outgroup taxon. Nucleotide divergence was determined using a Neighbor-Joining approach. The evolutionary distances were computed using the Kimura 2-parameter method [40] and are in the units of the number of base substitutions per site. The dendrogram clearly clustered the isolates into two branches supported by high values of bootstrap. All *A. tubingensis* isolates were grouped together whereas both *A. welwitschiae* and *A. niger*

isolates were clustered in the other branch. It is important to highlight that one *A. niger* isolate from oat (NIG-OAT-AN11) was early separated from the common branch supported by a bootstrap value of 94. The results show no apparent relationships with the source of isolation.

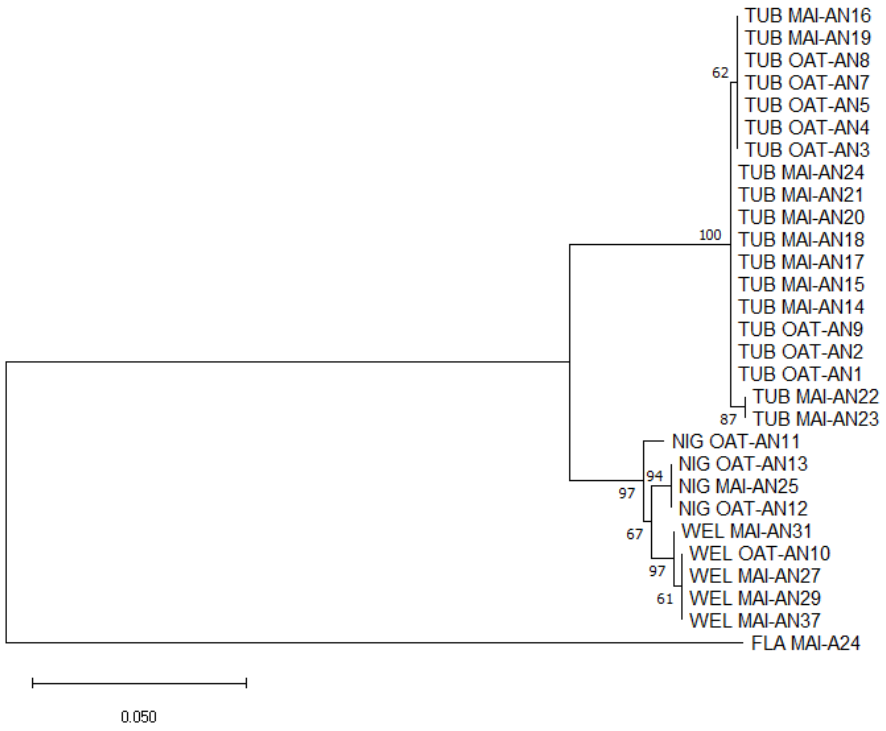

Figure 2. Phylogenetic analysis for 28 *Aspergillus niger* aggregate isolates from oat and maize, based on the sequence of a partial region of the calmodulin encoding gene (613 nucleotide positions). Tree was constructed by the Neighbor-Joining method with 1,000 bootstrap replicates, using the Kimura 2-parameter method. The optimal tree with the sum of branch length = 0.38120763 is shown.

The phylogenetic tree constructed using a partial region of the β-tubulin encoding gene of 38 isolates of *Aspergillus* section *Flavi* is shown in Figure 3. Nucleotidic divergence was determined using a Neighbor-Joining approach using the corresponding sequences of one *A. niger* isolate as outgroup. The evolutionary distances were computed using the Kimura 2-parameter method [40] and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.35). The cladogram separated the only *A. oryzae* isolate in a different branch although supported by a bootstrap value of 63. No apparent relationships were found between the identification of the isolates and their sources of isolation.

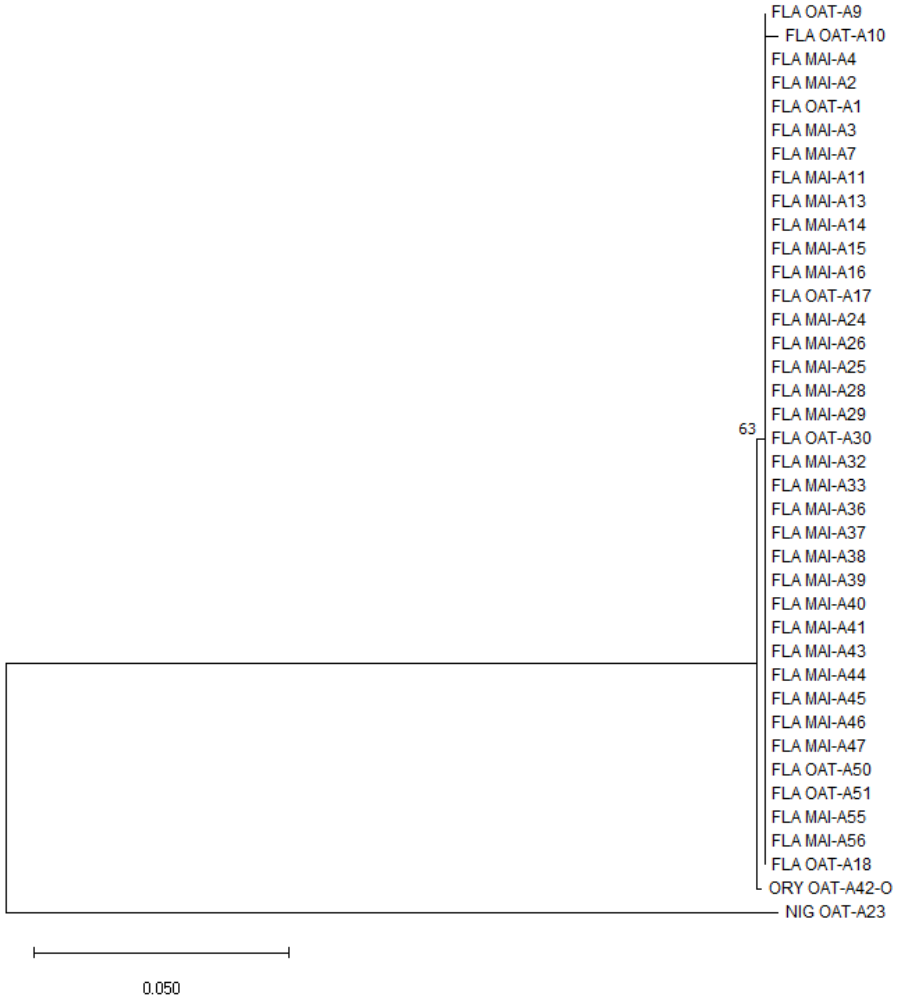

Figure 3. Phylogenetic analysis for 38 *Aspergillus* section *Flavi* isolates from oat and maize, based on the sequence of a partial region of the *β-tubulin* gene (369 nucleotide positions). Tree was constructed by the Neighbor-Joining method with 1,000 bootstrap replicates, using the Kimura 2-parameter method following a gamma distribution (shape parameter 0.35). The optimal tree with the sum of branch length = 0.30395258 is shown.

4. Discussion

Consumer demand for oat and maize-based food products has increased over the last decade. This fact has supposed an important rise in the production of these cereals, with the consequent spread of maize and oat cultivation areas [1]. Maize and oat-based products are consumed worldwide, since they are an important source of energy, vitamins and minerals [2]. These products are often contaminated by filamentous fungi which affect cereal grains, and may cause a rapid deterioration in their nutritional and organoleptic properties [18]. Moreover, filamentous fungi can also produce mycotoxins that can be carcinogenic or cause feed refusal and emesis [41], which suppose a risk for human and animal health [8].

The maximum levels of many mycotoxins in cereals and derivatives are strictly regulated in many regions including the European Union. However, there is no specific legislation setting the maximum levels of mycotoxins in oat and its derived

products. The presence of T-2 and HT-2 toxins and OTA in maize has not been regulated yet, due to the scarce number of studies regarding their relevance.

In this work, maize and oat samples have been evaluated in order to analyse the contamination levels of these products by mycotoxigenic fungi, and, therefore, to evaluate the potential risk posed by mycotoxins. Different samples were studied including maize and oat grains collected at harvest time as well as derived products marketed in Spanish retail stores.

There is little published information on the presence of these species and their mycotoxins in harvest oat and most of the studies were performed in Northern European areas. To our knowledge, this is the first extensive survey regarding the contamination of oat by mycotoxigenic species in Spain. Several authors reported high levels of contamination by *Fusarium* spp. in oat fields, being *F. langsethiae*, *F. poae* and *F. avenaceum* the most commonly detected species [13,42,43]. However, in our study the most frequently detected species in oat samples from Spain was *A. flavus* and the occurrence of *Fusarium* species was very low, and only *F. verticillioides*, *F. equiseti* and *F. graminearum* were demonstrated to be present in these samples. It is well known that *Fusarium* genus often contaminates cereal crops worldwide, although this contamination by some species and their mycotoxins is clearly influenced by regions and meteorological factors, such as temperature and rainfall during growing season [12,44]. *Fusarium* species are probably the most important source of mycotoxin contamination in small grains, such as oat, in Northern temperate regions, with abundant rainfull [42,45]. On the contrary, oat is grown under dry farming conditions in Spain, which could explain the presence of *A. flavus* in the oat samples harvested in Spain, since this species can grow under drier conditions than *Fusarium* spp. which need more humidity for their development [46].

A high percentage of harvest maize samples were positive for *F. verticillioides* (63 %), *F. proliferatum* (48 %) and *F. graminearum* (30 %). These results are in agreement with those reported by several authors who highlighted *F. verticillioides*, *F. proliferatum* and *F. graminearum* as the species of most concern in maize at pre-harvest in Europe [11, 47,48]. *Aspergillus flavus* (76 %) and *A. parasiticus* (7 %) were also detected in maize samples collected at harvest time. The detection rate of these species was quite similar to other studies performed in other countries; Giorni et al. [49] studied the contamination of maize intended for animal feed in Italy, and determined that *A. flavus* was much frequently occurring than *A. parasiticus* (93 and 7 % of contaminated samples, respectively). Wicklow et al. [50] and García-Díaz et al. (2020) also obtained similar data in USA and Spain, respectively [11]. A recent study has analysed the occurrence of mycotoxin in maize grains collected in Spain during the same period in which the present work was carried out [51]. The authors reported mycotoxin concentration values above the maximum limits allowed by the EU in maize for FB_1+FB_2, ZEA, AFB_1, and AFB_1+AFB_2. These results are in correlation to the expected mycotoxins taking into account the fungal species detected in the present work, which supported the hypothesis that to determine the presence of mycotoxigenic fungi can be useful to predict mycotoxin contamination.

Regarding maize and oat commercial products consumed in Spain, 60 % of commercial samples tested in this work were positive for contamination for at least one potentially mycotoxin-producing species, being the maize-based products the most contaminated. These data can be related to those reported by Serrano et al. [52], who studied mycotoxin contamination in oat and maize commercial samples from four countries of the Mediterranean region (Spain, Italy, Tunisia and Morocco). The mycotoxin incidence regarding the cereal type was higher in maize samples (39 % of positives for at least one toxin) than in the oat-based ones (17 %) in all areas studied. A current review published by Khaneghah et al. [53] reported that there is a high incidence of mycotoxins in cereal-based products distributed throughout Europe. Until now, several authors have studied the mycotoxin content in commercial cereal products suitable for human consumption, such as popcorn, cornflakes, oat-bran, flours, or babyfoods, and some of them found that mycotoxin levels exceed the European legislation [4,54-57]. In our study, the most frequently detected toxigenic species in commercial oat and maize based products marketed in Spain were *F. verticillioides* (44 % of contaminated samples), *F. proliferatum* (32 %), *A. flavus* (16 %) and *A. niger* (12 %) which are similar to the results obtained in oat and maize samples collected in Spanish fields. These species are able to produce different mycotoxins and their presence may be an indicator of a possible contamination risk by FUMs, AFs and OTA in cereal products, if drying and storage conditions are not adequate [58].

In general, the most frequently detected species in this study were *A. flavus, A. niger, F. verticillioides and F. proliferatum*. *Aspergillus flavus* is considered storage fungus, but it has been recently demonstrated that it can appear in maize fields from the beginning of the vegetative cycle [11]. *F. verticillioides* was detected in all types of samples, and its occurrence is more common in maize than in oat, whereas *F. proliferatum* was only detected in maize, in both field and commercial samples. It is well known that *F. verticillioides* and *F. proliferatum* are more competitive at high water activity levels than *A. flavus* or *A. niger* [59]; taking into account that maize is an irrigated crop, the grains may have higher water content than oat, which could explain why these species were present in the majority of maize samples.

As mentioned above, the contamination by potentially mycotoxin producing species is clearly influenced by regions where the crop is grown. For this reason, oat and maize samples were taken from the main cereal-producing areas of Spain, in order to know the current risk that might suppose the consumption of these cereals in this country. Different *Aspergillus* and *Fusarium* strains were isolated, and a phylogenetic study was carried out to find out if there was a genetic variability among them. The phylogenetic analysis based on Neighbor-Joining using the partial region gene of the *β-tubulin, calmodulin* or *ef-1α* revealed a consistent topology with the general genetic relationships for *Aspergillus* of section *Flavi*, *A. niger* aggregate and *Fusarium* species, respectively, although no phylogenetic relationships between the isolation sources, crop type or location were found. Although various authors have used these genes successfully in phylogenies constructed using isolates included in these genera, in our case these sequences appear to be non-informative to unravel evolutionary intraspecific relationships among *Fusarium* and *Aspergillus* isolates from Spain [60-63].

The analysis of *Aspergillus* strains of section *Flavi*, through the sequencing of the informative region of the genes encoding the β-tubulin, provided the identification of *A. flavus* and *A. oryzae*. The sequence of the ITS1-5.8S-ITS2 region is widely used in fungal identification, although it does not allow differentiate among different species of *Aspergillus* included in *Flavi* section [64]. In this context, the gene encoding *β-tubulin* is useful because the analysis of this partial sequence can differentiate morphologically identical *Aspergillus* taxa [60]. Similarly, *A. tubingensis*, *A. niger* and *A. welwitschiae* strains are morphologically indistinguishable species of *A. niger* aggregate. The analysis of the calmodulin encoding gene has been demonstrated to be able to discriminate among these closely related species. Recent studies carried out by our group, successfully applied the analysis of this gene to classify different isolates of the *A. niger* aggregate [61]. The phylogenetic study of this work showed that the three species were differentiated into two branches. One branch includes all *A. tubingensis* isolates wheras *A. niger* and *A. welwitschiae* clustered together in the other one. It is relevant to highlight that one *A. niger* isolate was separated (NIG-OAT-AN11) from the rest and the branch was supported by high bootstrap values. This strain has been previously reported as OTA producer, ability that is not widespread in *A. niger*. Therefore, this trait might be related to a phylogenetic significance but further studies would be necessary to unravel this hypothesis.

In the case of *Fusarium* isolates, a partial region of the *ef-1α* gene was sequenced and the phylogenetic study performed clearly clustered all *F. proliferatum* and *F. verticillioides* isolates into two different branches supported by high bootstrap values. Several authors have used this gene to differentiate between closely related *Fusarium* spp., as is the case with *F. verticillioides* and *F. proliferatum* [62,63].

The present work demonstrated the presence of potentially toxigenic species in maize and oat crops in the main cereal areas of Spain, as well as in commercial products distributed in Spain for human consumption. As mentioned above, the European Commission set the maximum allowed limits of some mycotoxins in cereals and their derivatives. Wide studies on the occurrence of mycotoxins in food products such as the presented in this work are very useful to consider if it is appropriate to modify the limits established by the legislation. The results obtained indicate that the risk posed by mycotoxin contamination of maize and oat in Spain is not high enough neither to plan a new legislation regarding oat nor to modify the current levels allowed in maize and its by-products.

5. Conclusion

Cereals such as maize and oat are basic sources of human and animal diet and their consumption have drastically increased due to new dietary habits. However, these cereals can be contaminated with potentially toxigenic species and mycotoxins which pose a high risk for food safety. In this study the presence of potentially toxigenic species of *Fusarium* and *Aspergillus* has been detected in oat and maize samples, both in field samples from the main cereal producing areas of Spain, and in cereal-based commercial samples marketed in Spain. The results indicated that the most frequent species detected in Spain were *Aspergillus flavus*, *A. niger*, *Fusarium*

verticillioides and *F. proliferatum*. The presence of these species can indicate a possible contamination by AF, OTA and FB in these products. These kind of studies are essential to provide information about the potential risk of food matrices to be contaminated by mycotoxins. Furthermore, our results indicate that it seems it is not necessary to amend the current European legislation; however, more studies are needed to relate the presence of mycotoxigenic fungi in maize and oat with their real content of mycotoxins.

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CHAPTER 3

A novel niosome-encapsulated essential oil formulation to prevent *Aspergillus flavus* growth and aflatoxin contamination of maize grains during storage.

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Abstract

Aflatoxin (AF) contamination of maize is a major concern for food safety. The use of chemical fungicides is controversial, and it is necessary to develop new effective methods to control *Aspergillus flavus* growth and, therefore, to avoid the presence of AFs in grains. In this work, we tested in vitro the effect of six essential oils (EOs) extracted from aromatic plants. We selected those from *Satureja montana* and *Origanum virens* because they show high levels of antifungal and antitoxigenic activity at low concentrations against *A. flavus*. EOs are highly volatile compounds and we have developed a new niosome-based encapsulation method to extend their shelf life and activity. These new formulations have been successfully applied to reduce fungal growth and AF accumulation in maize grains in a small-scale test, as well as placing the maize into polypropylene woven bags to simulate common storage conditions. In this latter case, the antifungal properties lasted up to 75 days after the first application.

Keywords

Essential oils; *Satureja montana*; *Origanum virens*; *Aspergillus flavus*; aflatoxin; corn; nanoparticles.

Key Contribution

A safe, ecofriendly, novel strategy was developed to prevent aflatoxin contamination of maize during storage. This method uses niosome-encapsulated EOs extracted from *Satureja montana* and *Origanum virens* and is able to control *Aspergillus flavus* growth for long periods.

1. Introduction

Aflatoxins (AFs) are secondary metabolites produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, and AFG_2) are the most important ones, with AFB_1 being the most toxic naturally occurring human carcinogen [1,2]. The International Agency for Research on Cancer (IARC) has classified the "naturally occurring mixes of aflatoxins" as Group 1 carcinogens in humans [3].

AFs contaminate a variety of staple crops including cereals (maize, sorghum, barley, oat, rye, rice, and wheat), soya, dry nuts (nuts, pistachios, almonds, and hazelnuts), cottonseed, coffee, cacao, and spices [4].

According to the Food and Agriculture Organization of the United Nations (FAO) [5], maize is one of the most important cereals, with an annual worldwide production of 1134 million tons in 2017, and most of it is intended for direct human and animal consumption. Moreover, maize and its derivatives are considered the main source of AFs worldwide [6]. For all these reasons, the European Union established strict regulations regarding maximum permitted AF levels for maize [7].

The impact of AF contamination of agri-food products is significant. It causes important economic losses because infected products cannot be sold and the contamination also raises veterinary and health costs. Establishing adequate controls to avoid AFs in the food chain is thus essential [8].

Different strategies to prevent AF contamination have been proposed to reduce fungal development in the field or during storage. Applying good agricultural practices and maintaining adequate humidity and temperature in silos are indispensable in reducing fungal growth and, therefore, mycotoxin contamination [9]. Chemical compounds are useful in preventing fungal growth and, for a long time, have been widely used both in the field and during storage to prevent mycotoxin contamination [10]. However, synthetic fungicides are in the spotlight and consumers are now demanding safer foodstuffs that are produced using sustainable and ecofriendly methods. The indiscriminate use of chemical fungicides has important drawbacks, including residue on grain that threatens human and animal health or causes extensive environmental contamination [11]. Moreover, the indiscriminate use of fungicides has caused an increased number of resistant isolates, which makes it very difficult to effectively control fungal growth [12].

The risks of using synthetic chemicals have increased public awareness and demand for safer and ecofriendly products and, in this context, natural plant extracts are now considered good alternatives. Essential oils (EOs) extracted from aromatic plants have demonstrated strong antibacterial, antifungal, and food preservative properties, together with low toxicity, fewer environmental effects, and wider public acceptance [13]. Many EO-based formulations are listed on the generally recognized as safe (GRAS) list, fully approved by the Food and Drug Administration, and are currently commercially available as food preservatives and/or agricultural supplements [14]. Several EOs have been reported to not only reduce growth in toxigenic fungal species, but also to interfere to some extent in mycotoxin biosynthesis.

Da Silva et al. [15] reported that *Rosmarinus officinalis* EO has a strong effect against *Fusarium verticillioides*, as it showed the ability to rupture the cell wall and inhibit the production of fumonisins. *Aspergillus flavus* growth and its ability to produce AFs were also significantly affected by treatment with *Origanum virens* and *Ageratum conyzoides* EOs in corn and soybeans [16], and using *Mentha spicata* EO in chickpeas [17].

Therefore, the use of EOs to prevent fungal growth during maize storage could be a sustainable solution to minimize food losses owing to mycotoxin contamination. However, their direct application in food products seems to be limited because of their high volatility, low water solubility, and susceptibility to oxidation [18]. To solve these problems, various encapsulation techniques have been developed that can preserve EOs through a physical or chemical interaction with a matrix that maintains the compounds for a longer time [19]. Encapsulation of EOs also increases stability against oxidation, which helps to prolong their antimicrobial activity [18,20]. Different methods of encapsulation have also been demonstrated to enhance the antifungal and antiaflatoxigenic properties when applied to control *A. flavus* [21].

These encapsulation particles form a protective film that isolates the nucleus that contains the active agent. The composition of the particles should be carefully chosen depending on the encapsulated compound. To date, several natural and synthetic matrices have been successfully used to encapsulate EOs including polyethylene, carbohydrates, proteins, lipids, and gum [22]. The choice of the encapsulation material is a crucial step in developing an appropriate application method for EOs. Different parameters should be taken into account such as the polarity, solubility, and volatility of the active compounds, as well as the composition of the food matrix [23]. Niosomes are lipid-based systems, composed by non-toxic self-assembly vesicles, with a single or multiple layered structure, which are able to encapsulate hydrophobic and hydrophilic compounds [24]. Niosomes are biodegradable, easily stored and handled, and present low toxicity, which are important advantages for their application in the food industry [24].

The aim of this work was to evaluate the in vitro antifungal and antitoxigenic effects of different aromatic plant EOs and to design an effective niosome-based encapsulation protocol to avoid AF contamination during maize storage.

2. Materials and Methods

2.1. Fungal strains and essential oils

All *Aspergillus flavus* strains used in this study were able to produce aflatoxins from group B (AFB_1 and AFB_2) and G (AFG_1 and AFG_2) and were isolated from wheat from Morocco (S.44-1) and maize from Spain (A7). The correct identification of these isolates was confirmed using species-specific PCR protocols [25].

The strains were stored as a spore suspension at −80 °C in 15 % glycerol (Panreac, Barcelona, Spain) until required. They were subcultured on potato dextrose agar (PDA, Pronadisa, Spain) and incubated at 28 °C for four days. The spore suspensions were prepared in sterile saline solution (9 g/L sodium chloride) (Merck,

Darmstadt, Germany) supplemented by Tween 80 0.5 % (Panreac, Spain). The spore concentration was determined using a Thoma counting chamber (Marienfeld, Lauda-Königshofen, Germany) and adjusted to a final concentration of 10^2 or 10^6 spores/mL depending on the assay.

The EOs tested were extracted from rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus vulgaris* L.), savory (*Satureja montana* L.), and three species of oregano (*Origanum virens* Hoffmanns. & Link, *O. majoricum* Camb., and *O. vulgare* L.). The EOs were provided by The Agricultural Research Centre of Albaladejito (Cuenca, Spain). Each species was processed in batches of 100–150 g of plant aerial parts, following the methodology proposed by the European Pharmacopoeia by hydrodistillation, in a Clevenger-type apparatus for 2 h. These EOs were analyzed in gas chromatograph equipped with a flame ionization detector (FID) and capillary column VF-5 of 60 mm × 0.25 mm, 5 % phenyl methyl siloxane. A temperature gradient of 70 to 240 °C was applied, with an increase of 3 °C per minute, maintaining the final temperature for 2 minutes. For the identification of the EO components, the relative retention times of standards and the corresponding Kovats indices were used. The quantification of the components was performed according to the areas of their chromatographic peaks.

These compounds were filtered (pore size 0.2 μm) (Fisherbrand, Shanghai, China) and stored at −20 °C in amber glass vials (Thermo Scientific, Madrid, Spain) until required.

2.2. Effectiveness of plant essential oils on fungal growth and aflatoxin production

The effect of EOs at different concentrations on *A. flavus* S.44-1 growth and its ability to produce AFs were evaluated on CYA medium (45.5 g/L of modified Czapek–Dox agar (Pronadisa, Spain), 5 g/L of yeast extract (Pronadisa, Spain)). EOs were diluted in polyethylene glycol 400 (PEG (Acros, Geel, Belgium)) and added to the medium to obtain final concentrations of 10, 100, 500, and 1000 μg/mL. The same amount of PEG was included in the control plates instead of EO. CYA plates supplemented with EOs were inoculated with 1.5 μL (4 mm of diameter) of a 10^6 spores/mL suspension on the center of the plate, and incubated at 28 °C for five days. All the conditions were tested in triplicate.

Fungal colony diameters were measured daily in two directions at right angles to each other until the medium was fully colonized (five days). Growth parameters were calculated from a linear model obtained by plotting the diameter (mm) against time (day). The parameters determined were λ, representing the lag phase (days prior to mycelial growth), and μ_{max}, representing the maximum growth rate (mm/day), for control plates and each EO concentration tested.

AFs were extracted from the plates after six days of incubation, as described elsewhere [26]. Three agar plugs were removed from the centre, medium, and outer edge of the colony and toxins were extracted with 1 mL of methanol (Merck, Spain). Samples were stored at –20 °C until analysis. AFs were measured by high performance liquid chromatography (HPLC) using the protocol described below.

2.3. *Effect of* Satureja montana *and* Origanum virens *essential oils encapsulated in niosomes on fungal growth and aflatoxin contamination*

2.3.1. Procedure for microencapsulation of essential oils

EOs extracted from *S. montana* and *O. virens* were encapsulated in non-ionic surfactant-based lipid vesicles (niosomes). These particles were prepared by Nanovex Biotechnologies S.L. (Oviedo, Spain) starting from 40 mL of each type of EO. Niosomes were obtained using the thin film hydration (TFH) method with homogenization and sonication to obtain niosomes with a good polydipersity index (PDI) and a particle size between 100 and 200 μm, with an EO concentration of 12 μL/mL.

The characterization of the niosomes was performed with a Zetasizer Nano ZS particle size analyzer (Malvern Panalytical Ltd., Malvern, UK), which uses dynamic light scattering (DLS) to determine particle size, and the M3-PALS technique to calculate the ζ-potential.

A nanoparticle tracking analysis (NTA) was performed using a nano sight particle tracking analyzer (Malvern Panalytical Ltd., Malvern, UK) to determine concentration and size distribution.

2.3.2. Effect of niosome-encapsulated essential oils on fungal growth and aflatoxin production on maize grains

Previously autoclaved maize grains were inoculated with *A. flavus*, strain A7. Then, 100 g of corn was immersed for 2 h in 100 mL of spore suspension 10^4 spores/mL to obtain a final concentration of 10^2 spores/g. Subsequently, the effect of niosome-encapsulated EOs was tested in a small-scale test in Petri dishes as well as in polypropylene woven bags simulating real storage conditions. At the beginning of the experiments, grain moisture was measured using a Hygropalm HP23A (Rotronic, Bassersdorf, Switzerland) and water activity was 0.95 in all cases.

2.3.2.1. Small-scale assays

Ninety millimeter Petri dishes were filled with crystalized potassium sulphate (Acros, Spain) to maintain a_w at 0.97 [27]. A 50 mm Petri dish containing 7 g of inoculated maize was placed inside the larger one. *Satureja montana* and *O. virens* EOs were applied directly to the grains or encapsulated in niosomes at 500 μg/g. Control assays, mock-inoculated with water, were also included. Incubation was performed at 28 °C and the effect of niosome-encapsulated EOs or those directly applied on maize grains was evaluated at 7 and 21 days.

After the incubation period, a sample of 3.5 g was taken from each treatment and a viable count was performed using serial decimal dilutions and inoculation on Rose Bengal with Chloramphenicol medium. Plates were incubated in darkness at 28 °C for two days. The fungal growth of maize grain was expressed as colony forming units per gram of maize (CFU/g).

Afterwards, another sample of 3.5 g was taken from each treatment, and shaken for 20 minutes with 35 mL of chloroform for AF extraction. AFB_1 was measured by thin layer chromatography (TLC), as described below.

2.3.2.2. Polypropylene woven bag assays

One-hundred grams of inoculated maize was placed in small polypropylene woven bags. Subsequently, niosome-encapsulated EOs (*S. montana* and *O. virens*) were added at a dose of 500 μg/g and mixed. Bags were incubated at room temperature in darkness for 90 days in independent plastic boxes for each treatment (40 × 40 × 30 cm). Inoculated maize grains without EOs were used as control. Temperature and relative humidity were registered using a data logger El-USB-1 (Easylog; LASCAR electronic, Salisbury, UK) every 8 h until the end of the assay.

After the incubation period, the bags were cut open and the maize was diluted in 900 mL of sterile saline solution (9 g/L) containing 0.05 % Tween 80. The mixes were incubated in an orbital shaker (140 rpm) at 4 °C for 60 minutes to release spores. Then, serial decimal dilutions and culture in Rose Bengal with Chloramphenicol were used to estimate fungal growth as CFU per gram of maize.

A sample of 14 g of corn was taken from each beaker, and shaken for 20 minutes with 35 mL of chloroform for AFB_1 extraction and subsequent evaluation by TLC, as described below.

2.4. Detection of mycotoxins

2.4.1. Detection of mycotoxins by high performance liquid chromatography (HPLC)

After AFB_1 extraction with methanol, mycotoxin measurements were performed in the "Laboratorio Arbitral Agroalimentario" (Madrid, Spain) following its standardized protocols. AF was measured by HPLC on a reverse phase C_{18} column (Inertsil ODS3; 5 μm, 4.6 mm × 250 mm; GL Sciences, Tokio, Japan) at 40 °C in a Waters chromatograph 515 HPLC coupled with a fluorescence detector 474 (Waters, Milford, MA, USA) at excitation and emission wavelengths of 362 and 435 nm, respectively. The mobile phase contained water, methanol, and acetonitrile (60:20:20), and the flow rate was 1 mL/minute. AF was eluted and quantified by comparison with a calibration curve generated from AF standards (OEKANAL®, Sigma–Aldrich, Steinheim, Germany). The detection limit of the technique was 2.5 ng/g.

2.4.2. Detection of mycotoxins by thin layer chromatography

After AFB_1 extraction with chloroform, samples were filtered using 0.45 μm syringe filters (Fisherbrand, Spain) and an aliquot of 1 mL was evaporated in a vacuum concentrator, Eppendorf™ Concentrator Plus with Pump and GB Plug (Fisher Scientific, Madrid, Spain).

Silica gel 60 chromatography plates (Merck, Germany) were used, and AFB_1 presence was determined according to the protocols described elsewhere [28, 29].

Samples and AF standards were re-suspended with 500 μL toluene/acetonitrile (95:5) (Panreac, Spain). Then, 10 μL of each sample was spotted on the plate. Toluene/acetone/acetonitrile (1:1:1 (LabKem, Barcelona, Spain)) was used as a mobile phase. Toxins were visualized under ultraviolet light (Spectronics, Westbury, NY, USA).

2.5. Statistical Analysis

Statistical analysis was performed on the effect of EOs encapsulated in niosomes with StatsGraphics Centurion XVII V.17.2.04 program (Statpoint Technologies Inc., Warrenton, VA, USA). The Shapiro–Wilk and Levene tests were used to check normality and homoscedasticity, respectively. Data were analysed using analysis of variance (ANOVA).

When data did not meet normality and homoscedasticity criteria, a non-parametric Kruskall–Wallis test was performed. These analyses were performed using the software InfoStat/E 2011 (FCA, Córdoba, Argentina). This was necessary in the case of the fungal growth and aflatoxin production variables indicated in Section 2.2 of Material and Methods. In all cases, the significance level was set at $p < 0.05$.

3. Results

3.1. The efficacy of plant essential oils against fungal growth and mycotoxin production

Figure 1 and Figure 2 show the results for the *Aspergillus flavus* growth rate (μ, mm/day) and the lag phase prior to growth (λ, h), respectively, in CYA (Czapek Yeast Autolysate Agar) plates supplemented with different essential oils (EOs) (*Rosmarinus officinalis, Thymus vulgaris, Satureja montana, Origanum virens, O. majoricum*, and *O. vulgar*e) at several different concentrations (0, 10, 100, 500, and 1000 μg/mL).

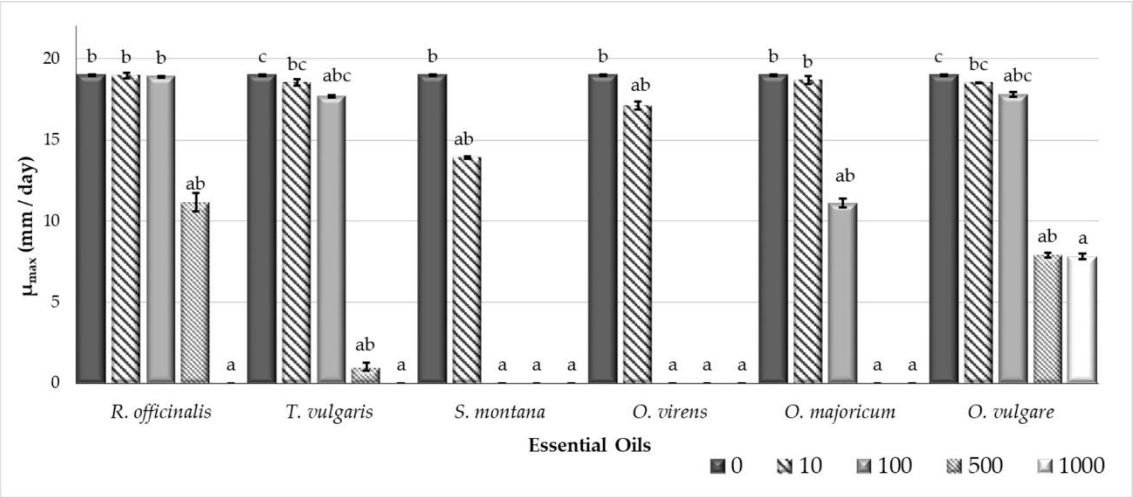

Figure 1. *Aspergillus flavus* S.44-1 growth rate (mm/day) at different concentrations (0, 10, 100, 500, and 1000 μg/mL) of essential oils (*R. officinalis, T. vulgaris, S. montana, O. virens, O. majoricum*, and *O. vulgare*). Each value is the mean of three replications and the thin vertical bars represent the standard error of the corresponding data. Groups with the same letter are not significantly different ($p > 0.05$).

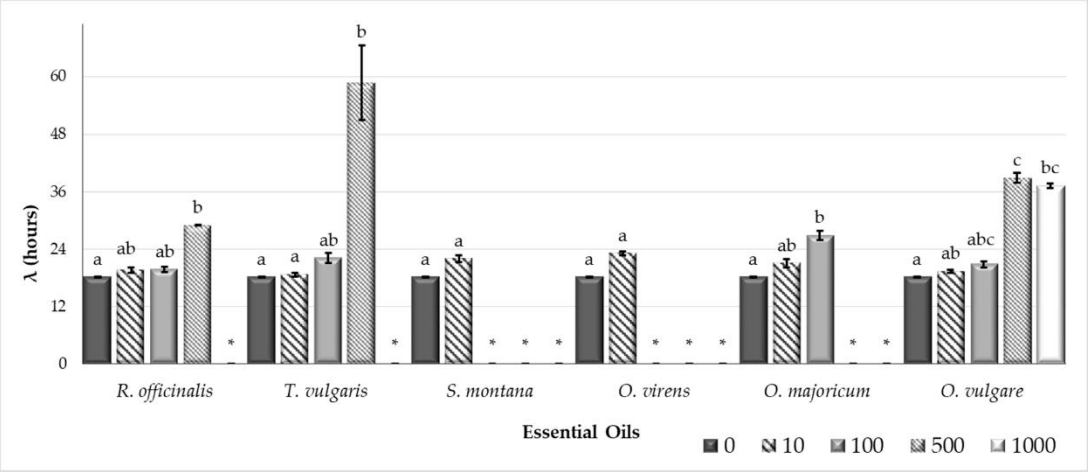

Figure 2. *Aspergillus flavus* S.44-1 lag phase (h) at different concentrations (0, 10, 100, 500, and 1,000 μg/mL) of essential oils (*R. officinalis, T. vulgaris, S. montana, O. virens, O. majoricum, and O. vulgare*). Each value is the mean of three replications and the thin vertical bars represent the standard error of the corresponding data. Groups with the same letter are not significantly different ($p > 0.05$). * No data.

All EOs tested had a significant effect on the *A. flavus* growth rate at the maximum concentration (Figure 1). Fungal growth was completely inhibited, except for the *O. vulgare* EO. For the EOs of *O. virens* and *S. montana*, total inhibition was reached at 100 μg/mL. *Thymus vulgaris* and *O. majoricum* EOs also showed reductions of at least 95 % at 500 μg/mL.

The lag phase got longer as the EO concentration increased (Figure 2). It was not possible to calculate the lag phase when the EO treatment completely inhibited growth in the plates.

Aflatoxin production (AFB_1, AFB_2, AFG_1 y AFG_2) was significantly reduced compared with the control group in all treatments at the highest concentration tested (1000 μg/mL). No AFs were detected at these concentrations (Table 1) in any case, except for the *O. vulgare* EO treatment, which achieved reductions of more than 97 % in all toxins.

Table 1. Aflatoxin (AF) concentration (B_1, B_2, G_1, and G_2) in Czapek Yeast Autolysate Agar (CYA) plates supplemented with different concentrations (0, 10, 100, 500, and 1000 μg/mL) of essential oils (EOs) (*R. officinalis, T. vulgaris, S. montana, O. virens, O. majoricum*, and *O. vulgare*). Each value is the mean of three replications ± standard error. Groups with the same letter are not significantly different ($p > 0.05$).

Essential oils	μg/mL	B_1 (μg/g agar)	B_2 (μg/g agar)	G_1 (μg/g agar)	G_2 (μg/g agar)
R. officinalis	0	10.754 ± 0.925 ^{c}	0.201 ± 0.021 ^{c}	0.485 ± 0.055 ^{c}	0.088 ± 0.014 ^{b}
	10	5.205 ± 1.033 ^{bc}	0.100 ± 0.022 ^{bc}	0.213 ± 0.045 ^{bc}	ND ^{a}
	100	5.223 ± 0.171 ^{abc}	0.110 ± 0.006 ^{bc}	0.216 ± 0.012 ^{abc}	ND ^{a}
	500	1.090 ± 0.152 ^{ab}	0.017 ± 0.002 ^{ab}	0.058 ± 0.007 ^{ab}	ND ^{a}
	1000	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
T. vulgaris	0	10.754 ± 0.925 ^{b}	0.201 ± 0.021 ^{b}	0.485 ± 0.055 ^{b}	0.088 ± 0.014 ^{b}
	10	7.040 ± 0.977 ^{b}	0.122 ± 0.019 ^{ab}	0.256 ± 0.059 ^{ab}	ND ^{a}
	100	5.994 ± 0.554 ^{ab}	0.117 ± 0.019 ^{ab}	0.264 ± 0.069 ^{ab}	ND ^{a}
	500	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
	1000	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
S. montana	0	10.754 ± 0.925 ^{b}	0.201 ± 0.021 ^{b}	0.485 ± 0.055 ^{b}	0.088 ± 0.014 ^{b}
	10	8.270 ± 0.686 ^{ab}	0.151 ± 0.011 ^{ab}	0.314 ± 0.029 ^{ab}	ND ^{a}
	100	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
	500	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
	1000	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
O. virens	0	10.754 ± 0.925 ^{b}	0.201 ± 0.021 ^{bc}	0.485 ± 0.055 ^{b}	0.088 ± 0.014 ^{b}
	10	10.520 ± 1.334 ^{b}	0.245 ± 0.039 ^{c}	0.508 ± 0.065 ^{b}	ND ^{a}
	100	0.033 ± 0.044 ^{ab}	0.003 ± 0.000 ^{ab}	0.004 ± 0.002 ^{ab}	ND ^{a}
	500	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
	1000	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
O. majoricum	0	10.754 ± 0.925 ^{b}	0.201 ± 0.021 ^{ab}	0.485 ± 0.055 ^{b}	0.088 ± 0.014 ^{b}
	10	10.939 ± 0.210 ^{b}	0.234 ± 0.008 ^{b}	0.611 ± 0.057 ^{b}	ND ^{a}
	100	7.999 ± 0.628 ^{ab}	0.186 ± 0.022 ^{ab}	0.375 ± 0.028 ^{ab}	ND ^{a}
	500	0.003 ± 0.000 ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
	1000	0.008 ± 0.008 ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
O. vulgare	0	10.754 ± 0.925 ^{c}	0.201 ± 0.021 ^{b}	0.485 ± 0.055 ^{b}	0.088 ± 0.014 ^{b}
	10	10.143 ± 0.860 ^{bc}	0.192 ± 0.015 ^{b}	0.475 ± 0.056 ^{b}	ND ^{a}
	100	7.867 ± 0.409 ^{abc}	0.168 ± 0.015 ^{ab}	0.466 ± 0.080 ^{b}	ND ^{a}
	500	0.738 ± 0.080 ^{ab}	0.012 ± 0.002 ^{a}	0.043 ± 0.003 ^{ab}	ND ^{a}
	1000	0.298 ± 0.068 ^{a}	0.004 ± 0.003 ^{a}	0.014 ± 0.004 ^{a}	ND ^{a}

ND: non detected.

AFB_1 production was significantly affected at 500 μg/mL, with reductions of nearly 100 % in all of the EOs tested. The same results were obtained when *O. virens* and *S. montana* EOs were applied at 100 μg/mL.

AFB_2 production was also significantly reduced by least 90 % compared with the control plates after treatment with *R. officinalis, T. vulgaris, O. vulgare*, and *O. majoricum* EOs at 500 μg/mL. The most effective treatments, reaching complete inhibition of AFB_2 production at 100 μg/mL, were *O. virens* and *S. montana* EOs.

Aflatoxin G_1 was not detected on *S. montana* and *O. virens* at 100 μg/mL. The rest of the EOs showed a reduction greater than 80 % at 500 μg/mL. In all cases, AFG_2 concentration was below the detection limit (<0.0025 μg/g agar), except in the case of control plates without treatment.

3.2. Techniques for the application of essential oils to prevent fungal growth and mycotoxin production

The main nanocapsule characteristics of the *O. virens* and *S. montana* niosomes can be found in Table 2. According to these data, both encapsulation processes yield high quality niosomes with low aggregation of nanoparticles. The particle size of both samples was approximately 140 nm with a polydipersity index (PDI) of 0.251 and a ζ-potential of –14 mV.

Table 2. Characterization of *O. virens* and *S. montana* essential oil particles encapsulated in niosomes.

	ZETASICER			**NANOSIGHT**	
	PDI	Z-AVERAGE (nm)	POTENCIAL-ζ (mV)	Size (nm)	CONCENTRATION (particle/mL)
O. virens	0.251 ± 0.019	156.2 ± 3.9	- 14.5 ± 0.5	142.4 ± 1.0	$(2.96 \pm 0.12)10^{14}$
S. montana	0.251 ± 0.011	153.3 ± 2.8	- 14.6 ± 2.3	140.6 ± 3.8	$(1.86 \pm 0.07)10^{14}$

PDI: polydipersity index.

3.2.1 Small-Scale Assay

Satureja montana and *O. virens* EOs applied by direct contact to artificially inoculated maize grains and incubated at 28 °C reduced *A. flavus* growth compared with the control group without EOs after seven days of incubation, although no significant differences were observed (Figure 3a). When these EOs were applied in their niosome-encapsulated form and incubated for seven days, colony forming units (CFU) per gram values were slightly increased, although no significant differences were found. When incubation time was extended to 21 days, the opposite effect was observed (Figure 3b). EOs applied by direct contact seemed to lose their effect over time. However, when these compounds were encapsulated in niosomes, *S. montana* EO reduced fungal growth by 58 % and, in the case of *O. virens*, this reduction was 32 % with respect to the control group.

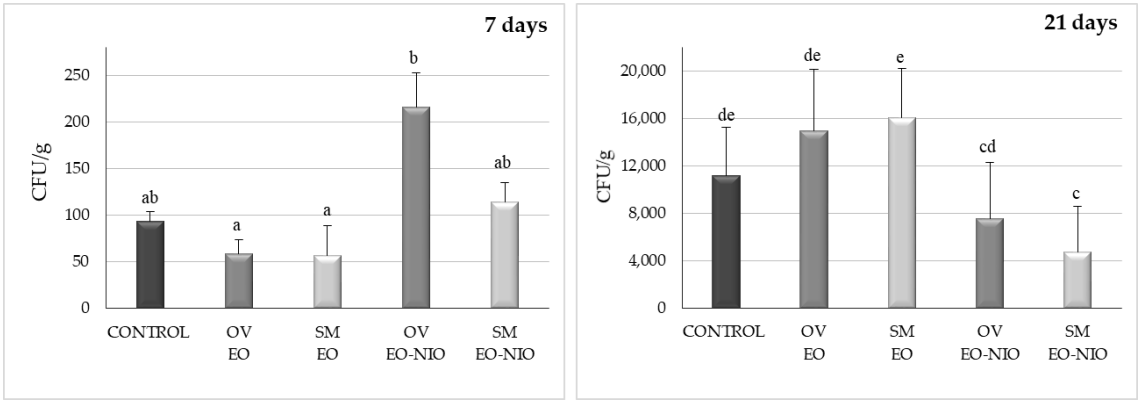

Figure 3. Effect of *S. montana* (SM) and *O. virens* (OV) by direct contact (essential oil, EO) and immobilized in niosomes (EO-NIO) on corn grains inoculated with *A. flavus*, incubated for 7 (**a**) and 21 days (**b**). Each values is the mean of three replications and the thin vertical bars represent the standard error of the corresponding data. Groups with the same letter are not significantly different ($p > 0.05$). CFU, colony forming units.

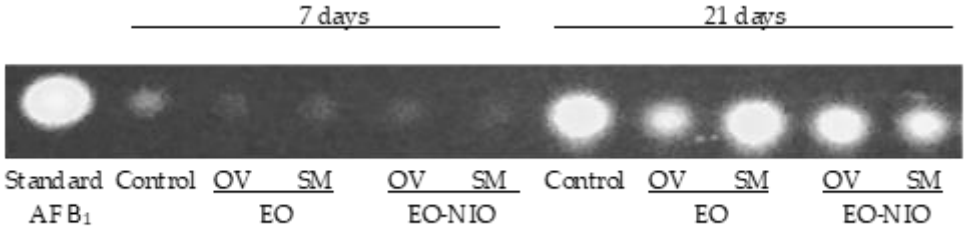

Figure 4. Effect of *S. montana* (SM) and *O. virens* (OV) by direct contact (EO) and encapsulated in niosomes (EO-NIO) on aflatoxin (AF) B_1 concentration of corn grains inoculated with *A. flavus*, incubated for 7 and 21 days. The standard corresponds to the application of purified AFB_1 (0.05 mg/mL).

AFB_1 production was low at the short incubation time (7 days) and very significant for longer periods (21 days) (Figure 4). In the thin layer chromatography (TLC) analysis, the intensity and thickness of the fluorescent band is related to AFB_1 concentration. Therefore, in the seven-day long assay, AFB_1 concentration was reduced in all cases with respect to control. When plates were incubated over 21 days, a reduction in AFB_1 concentration was observed in plates treated directly with *O. virens* EO as well as both EO niosome-encapsulated. However, no differences were found after *S. montana* EO application.

3.2.2. Polypropylene woven bags assays

Figure 5 shows the results in CFU/g of *A. flavus* inoculated corn stored in polypropylene woven bags at all incubation times and after treatment with EOs encapsulated in niosomes.

After 45, 60, and 75 days of incubation, both of the EOs encapsulated in niosomes were able to control fungal growth, with a maximum reduction of up to 79 % and 69 % for *S. montana* and *O. virens* EOs, respectively. However, this effect seems to be lost over time and no significant differences with respect to the control group were found after 90 days of incubation.

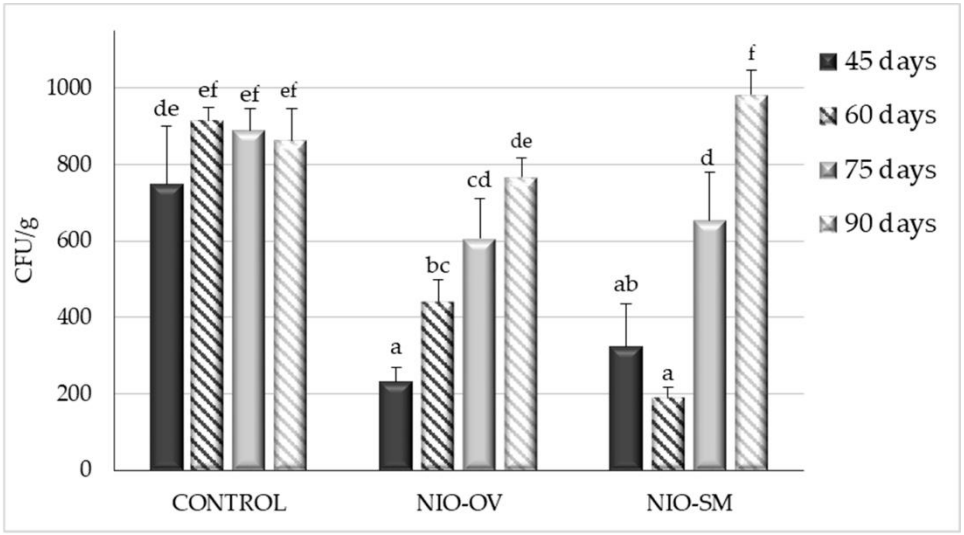

Figure 5. Effect of *S. montana* (NIO-SM) and *O. virens* (NIO-OV) EO encapsulated in niosomes on *A. flavus* growth in corn grains incubated for 45, 60, 75, and 90 days. Each value is the mean of three replications and the thin vertical bars represent the standard error of the corresponding data. Groups with the same letter are not significantly different ($p > 0.05$).

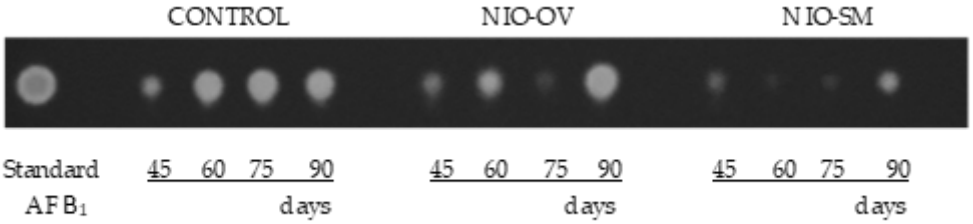

Figure 6. AFB_1 detection by thin layer chromatography (TLC) in polypropylene woven bags inoculated with *A. flavus* after *S. montana* (NIO-SM) and *O. virens* (NIO-OV) niosome treatment of corn incubated for 45, 60, 75, and 90 days. The intensity and thickness of the fluorescent band are related to the concentration of toxin. The standard corresponds to the application of purified AFB_1 (0.05 mg/mL).

The results regarding AFB_1 detected on inoculated control bags or after niosome-encapsulated EO treatments are shown in Figure 6. The band intensity of each treatment was apparently lower with respect to their corresponding control in all cases except for NIO-OV after 90 days of incubation when a slight increase in production was detected. Treatment using niosome-encapsulated *S. montana* EO was the most effective to control AFB_1 production by *A. flavus*.

Temperature and humidity were recorded during the experiment with ranges of 25–27 °C and 75–85 %, respectively.

4. Discussion

Aflatoxin (AF) presence poses a high risk to food security and most countries have established maximum levels of these contaminants allowed in food products [3]. Appropriate control mechanisms are needed to keep these toxins from entering the food chain. In recent years, essential oils (EOs) have come to be considered as a safe, ecofriendly, renewable, and easily biodegradable option to be used as a food supplement [13]. Moreover, many EOs have been described as potent antifungal

compounds that are able to interfere in mycotoxin synthesis [15,30–32]. In this work, we selected EOs extracted from *Rosmarinus officinalis*, *Thymus vulgaris*, *Satureja montana*, *Origanum virens*, *O. majoricum*, and *O. vulgare* to determine if they were able to control *Aspergillus flavus* growth and if they reduced AF production by this fungus. To some extent, all of the EOs tested modified the fungal growth rate and extended the lag phase at high concentrations. However, at lower doses, the EOs extracted from *S. montana* and *O. virens* were the most effective at reducing both fungal growth and AF production, and they were selected to perform subsequent studies. Chromatographic characterization of the EOs used in this study were carried out in the Agricultural Research Centre of Albaladejito (data not shown) and the results revealed that *S. montana* and *O. virens* EOs are highly rich in carvacrol and thymol, respectively. These compounds have been reported to be able to interact with the cell membrane, disrupt cell permeability, and produce cell death [33]. Pure extracts of both carvacrol and thymol have been reported to inhibit the growth of important mycotoxin-producing species such as *A. niger*, *A. flavus*, *A. ochraceus*, and *F. graminearum* [34,35].

Different authors consider that EO-based formulations could be safe, ecofriendly preservatives to avoid post-harvest losses due to mycotoxin contamination [14]. Therefore, taking into account the potent antifungal properties of EOs, many studies have focused on developing successful application protocols to minimize their drawbacks, which limit their direct use in food products [18] and, therefore, it is essential to protect them to extend their shelf life and activity [19]. The controlled liberation of EOs and their encapsulation in nanoparticles made of different materials are considered a good option [19,23]. These technologies attempt to reduce the rapid loss of their active principles. In general, EOs are a complex mix of lipophilic compounds and, therefore, lipid nanoparticle systems such as liposomes are the most appropriate [23,36]. In our study, the small-scale experiments showed that the effectiveness of EOs to control *A. flavus* growth diminished over time. However, when both EOs were encapsulated in niosomes, a significant reduction in fungal viable counts with respect to the untreated control group were found even after 21 days of incubation. Hence, it seems that the niosome-based nanoparticles were able to reduce the loss of the EOs' active principles and produce a controlled release of their compounds. Similar results were obtained in the larger-scale experiments using maize stored in polypropylene woven bags. This type of storage is very common in African countries because it offers low-cost protection for grain from pests. However, a higher contamination by toxigenic fungi and mycotoxins in the grains has been reported owing to the change in moisture content, which increases the relative humidity inside the bags [37]. Even under the worst conditions for maize storage, both of the EOs encapsulated in niosomes were able to control fungal development, significantly reducing aflatoxin levels, and their effect was extended for up to 75 days. Hence, these promising approaches might be useful to prevent AF contamination under more appropriate storage conditions such as PICS (Purdue improved crop storage) bags or directly in silos.

The use of EOs in the agri-food industry is not a safety concern because several studies have ensured that they are safe as food additives and many of them are included in the GRAS list [14]. However, data are scarce regarding the effects that vesicle materials might have on human health and it is essential to carry out ecotoxicity

studies to assess the impact of encapsulation matrices [19]. The niosome vesicles used in this work are commercially available and their non-toxic properties have been fully demonstrated. Moreover, they have been approved as a good option for the development of nanoparticles to improve medical therapies, including the controlled delivery of drugs or even vaccine antigens [24].

EO-based formulations need to overcome several tests before their application in food systems, as some active components can interact with food matrix components [14,38]. AFs often occur in maize, one of the most important basic cereal products worldwide for food and feeds [6]. In the present work, *S. montana* and *O. virens* EOs encapsulated in niosomes were directly applied to control *A. flavus* growth and its mycotoxigenic potential in artificially contaminated maize as a preliminary step to optimizing their application. EOs' release was effective over time in both small-scale tests and simulated storage conditions and, therefore, no interactions with the components of the maize seemed to occur. It would be interesting to apply this newly developed technology to other food matrices often contaminated with AFs to confirm that this effect could be extrapolated to other products.

5. Conclusion

In this work, we proposed a novel niosome-based EO product that was successfully applied in polypropylene woven bags simulating common storage conditions of maize. The involvement of the company Nanovex Biotechnologies S.L. guarantees a correct and standardized encapsulation protocol and the reduction of problems that might arise during product formulation. The presence of encapsulated EOs in the bags significantly reduced *A. flavus* development and the effect was observed until 75 days after inoculation. The effect of this formulation could be easily maximized by applying the products regularly during maize storage, that is, every 45 days. Regularly scheduled application, together with good agricultural practices and the maintenance of adequate storage conditions, may be a sustainable way to avoid the occurrence of aflatoxins in stored maize.

Author Contributions: All authors conceived the experimental design. M.G.-D., J.G.-S., and B.P. helped with laboratory analysis. M.G.-D. and J.G.-S. performed statistical analysis and wrote the original draft. B.P. and C.V. reviewed and edited the manuscript. All authors read and approved the final version of the document.

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Conflicts of Interest: The authors declare no conflict of interest.

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SUPPLEMENTARY INFORMATION

APPENDIX I. Application of hydrolates to control fungal growth and mycotoxin production by *Aspergillus flavus*.

1. Introduction

Aromatic plant extracts, and in particular essential oils (EOs) have been widely explored for their antimicrobial and antioxidant properties [1]. However, few studies have been performed regarding the characteristics of hydrolates (HLs). These compounds, also known as hydrosols or floral waters, are aqueous solutions derived from the hydro-distillation of EOs, and contain traces of EOs and other water-soluble compounds [2]. Hydrolates are easy and inexpensive to produce and are practically innocuous for human health [3]. These aromatic plant by-products have antifungal, antibacterial, and antioxidant properties [4-7] and they have been proposed as phytosanitary products [8].

In this work, six HLs were tested in vitro to evaluate their effect on growth and aflatoxin (AF) production by *Aspergillus flavus*. The experimental procedures were similar to those explained in the present chapter (section 2.2 of Materials and Methods), using HLs instead of EOs, in order to be able to compare the antifungal and antitoxigenic ability of both plant extracts.

2. Materials and Methods

Six HLs obtained from rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus vulgaris* L.), savory (*Satureja montana* L.), and three species of oregano (*Origanum virens* Hoffmanns. & Link, *O. majoricum* Camb., and *O. vulgare* L.) were tested in vitro towards *A. flavus* to evaluate their effect on its growth and its ability to produce AF. The *A. flavus* strain used was S.44-1, which is able to synthetize AFB_1, B_2, G_1 and G_2 and it was isolated from Moroccan wheat by our group.

The HLs were provided and characterized by The Agricultural Research Centre of Albaladejito (Cuenca, Spain). These extracts were the residues of the hydro-distillation of the same aromatic plants tested in previous works carried out during this Thesis (present chapter). Chromatographic characterization of the HLs and EOs used in this Thesis are shown in Table 1. These extracts were filtered (pore size 0.2 μm (Fisherbrand, Shanghai, China)) and stored at −20 °C in amber glass vials (Thermo Scientific, Madrid, Spain) until required.

Table 1. Chemical compounds present in the hydrolates (HLs) and essential oils (EOs) of *Rosmarinus officinalis, Thymus vulgaris, Origanum majoricum, O. vulgare O. virens and Satureja montana*. The detection time and the compounds detected (> 1 %) are shown. Compounds with percentages >10 % are highlighted in bold.

Time						*Rosmarinus officinalis*		*Thymus vulgaris*		*Origanum majoricum*		*Origanum vulgare*		*Origanum virens*		*Satureja montana*	
(minutes)	**Compound**	**Chemical composition**				EOs	HLs	EOs	HLs	EOs	HLs	EOs	HLs	EOs	HLs	EOs	HLs
11.04	alpha-thujone	ketone	mono terpene	$C_{10}H_{16}O$		-	-	-	-	1.2	-	-	3.9	-	-	2	-
11.39	alpha-pinene		mono terpene	$C_{10}H_{16}$		16.7	7.4	5.1	-	-	-	-	-	-	-	1.5	-
12.01	camphene		mono terpene	$C_{10}H_{16}$		9.5	-	8.9	-	-	-	-	-	-	-	-	-
12.63	sabinene		mono terpene	$C_{10}H_{16}$		-	1.0	2.5	-	6.7	-	3.3	-	-	-	1.0	1.9
12.98	beta-pinene + myrcene		mono terpene	$C_{10}H_{16}$ + $C_{10}H_{16}$		9.8	4.0	8.1	-	2.6	-	2.2		2.4	1.3	5.7	-
13.81	delta-3-carene		mono terpene	$C_{10}H_{16}$		-	-	-	-	-	-	-	1.5	-	-	-	-
14.18	alpha-terpinene		mono terpene	$C_{10}H_{16}$		-	-	-	-	6.1	-	1.6	-	4.5	2.1	3.2	-
14.49	para-cymene		mono terpene	$C_{10}H_{14}$		1.9	-	1.4	-	3.1	-	2.7	-	3.6	1.6	**12.6**	-
14.64	limonene		mono terpene	$C_{10}H_{16}$		5.8	2.2	2.5	-	3.3	-	7.8	-	-	-	1.6	-
14.91	eucalyptol	alcohol	mono terpene	$C_{10}H_{18}O$		**13.8**	**14.3**	**35.7**	**40.6**	-	-	-	-	-	-	-	-
15.02	e-beta-ocimene		mono terpene	$C_{10}H_{16}$		-	-	1.4	-	-	-	**24.8**	-	-	-	0.1	-
15.70	gamma-terpinene		mono terpene	$C_{10}H_{16}$		-	-	**2.2**	-	**19.0**	-	**10.4**	-	**45.9**	-	**22.5**	1.0
16.18	cis-sabinene hydrate		mono terpene	$C_{10}H_{18}$		-	-	-	-	2.9	-	1.5	-	-	**16.2**	-	1.0
16.70	terpinolene		mono terpene	$C_{10}H_{16}$		-	1.7	-	-	1.7	-	-	-	-	-	-	
17.02	linalool	alcohol	mono terpene	$C_{10}H_{18}O$		1.3	1.0	1.7	1.0	1.4	1.5	-	-	-	-	1.6	1.8
18.04	beta-thujone	ketone	mono terpene	$C_{10}H_{16}O$		-	1.6	0.1	-	1.1	1.7	-	-	-	-	-	-
19.21	camphor	ketone	mono terpene	$C_{10}H_{16}O$		**22.9**	**23.6**	**11.7**	**13.7**	-	-	-	-	-	-	-	-
19.94	beta-pinene-oxide	oxide	mono terpene	$C_{10}H_{16}O$		-	-	-	1.5	-	-	-	-	-	-	-	-
20.13	borneol	alcohol	mono terpene	$C_{12}H_{20}O_2$		3.2	3.2	4.8	3.8	-	-	-	-	-	-	1.4	2.4
20.32	terpinen-4-ol	alcohol	mono terpene	$C_{10}H_{18}O$		1.0	1.2	2.8	2.7	**13.5**	**55.1**	2.2	5.5	-	1.0	1.0	1.3
20.80	alpha-terpineol	alcohol	mono terpene	$C_{10}H_{18}O$		1.3	1.8	2.8	3.9	1.9	6.7	-	-	-	-	-	-
20.97	myrtenol	alcohol	mono terpene	$C_{10}H_{16}O$		0.1		-	-	-	-	-	-	-	-	1.3	-
21.28	verbenone	ketone	mono terpene	$C_{10}H_{14}O$		1.6	7.8	-	-	-	-	-	1.9	-	-	-	-
22.16	nerol	alcohol	mono terpene	$C_{10}H_{18}O$		-	-	-	-	3.1	-	-	-	1.3	-	-	-
22.43	linalyl acetate		acetate	$C_{12}H_{20}O_2$		-	-	-	-	2.5	-	-	-	-	-	-	-
23.95	bornyl acetate		acetate	$C_{12}H_{20}O_2$		1.2	-	-	-	-	**16.2**	1.0	1.3	-	**45.5**	-	2.3
23.97	thymol	phenol	mono terpene	$C_{10}H_{14}O$		-	-	-	-	7.2	-	-	-	**20.9**	-	1.9	-
24.42	carvacrol	phenol	mono terpene	$C_{10}H_{14}O$		-	-	-	-	1.7	4.7	-	1.1	-	-	**34.5**	**77.9**
27.48	beta elemene		sesqui terpene	$C_{15}H_{24}$		-	-	-	-	-	-	1.0	1.8	-	-	-	-
28.70	trans-caryophyllene		sesqui terpene	$C_{15}H_{24}$		-	-	-	-	1.9	-	**11.5**	-	5.1	-	-	-
28.87	alpha-bergamotene		sesqui terpene	$C_{15}H_{24}$		-	-	-	-	-	-	-	-	-	-	-	1.3
29.84	beta-farnesene		sesqui terpene	$C_{15}H_{24}$		-	-	-	-	-	-	1.4	-	1.0	-	-	-
30.60	valencene		sesqui terpene	$C_{15}H_{24}$		-	-	-	-	1.0	-	**10.0**	-	2.9	-	-	-
31.05	gamma-muurolene		sesqui terpene	$C_{15}H_{24}$		-	-	-	-	1.4	-	-	-	-	-	-	-
31.08	bicyclogermacrene		sesqui terpene	$C_{15}H_{24}$		-	-	-	-	-	-	1.7	-	2.0	-	1.0	-
33.58	spa thulenol		sesqui terpene	$C_{15}H_{24}$		-	-	-	-	-	-	-	1.2	-	-	-	-
33.82	caryophyllene oxide	oxide	sesqui terpene	$C_{15}H_{24}O$		-	-	-	-	-	-	2.0	-	-	-	-	-

The effect of HLs at different concentrations on *A. flavus* S.44-1 growth and its ability to produce AFs were evaluated on CYA medium following the protocol described in this chapter (section 2.2 "Effectiveness of plant essential oils on fungal growth and aflatoxin production"; and section 2.4.1 "Detection of mycotoxins by HPLC"). The HLs were diluted in water and added to the medium to obtain final concentrations of 50,000 and 75,000 μg/mL. All the conditions were tested in triplicate.

The statistical analysis of the variables fungal growth and AF concentration was performed, using InfoStat/E 2011 program (FCA, Córdoba, Argentina). In all cases, the significance level was set at $p < 0.05$.

3. Results

The results regarding the *A. flavus* growth rate, the lag phase prior to growth and AF concentrations in CYA plates supplemented with the HLs at different concentrations are shown in Figure 1, Figure 2 and Table 2, respectively. These variables did not meet normality and homoscedasticity criteria; therefore, non-parametric Kruskall-Wallis analyses were performed independently for each HL.

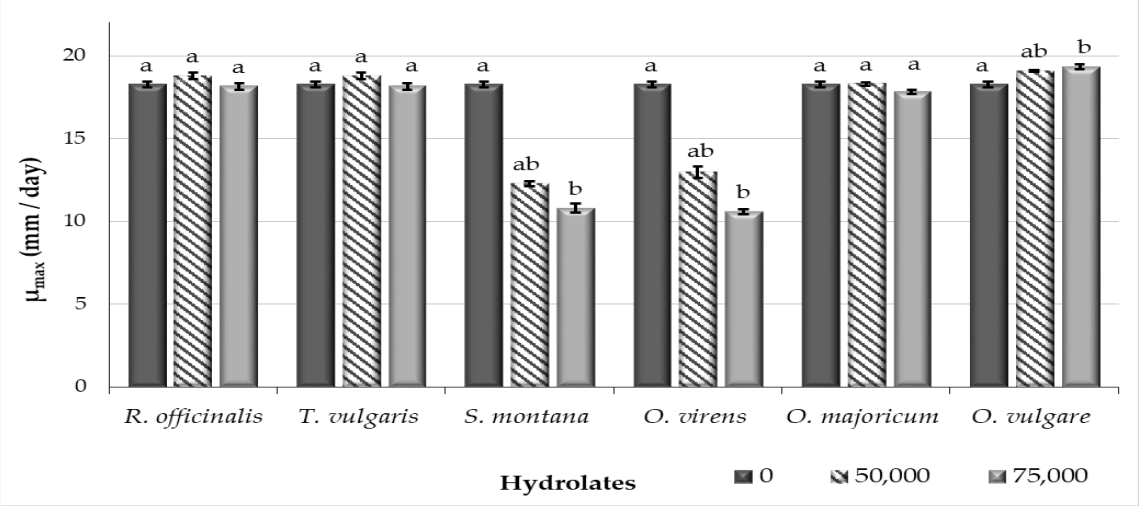

Figure 1. *Aspergillus flavus* S.44-1 growth rate (mm/day) at different concentrations (0, 50,000 and 75,000 μg/mL) of hydrolates (*R. officinalis*, *T. vulgaris*, *S. montana*, *O. virens*, *O. majoricum* y *O. vulgare*). Each value corresponds to the mean of three replicates and the thin vertical bars represent the standard error of the data. Groups with the same letter are not significantly different ($p > 0.05$). Statistical analysis was performed independently for each compound.

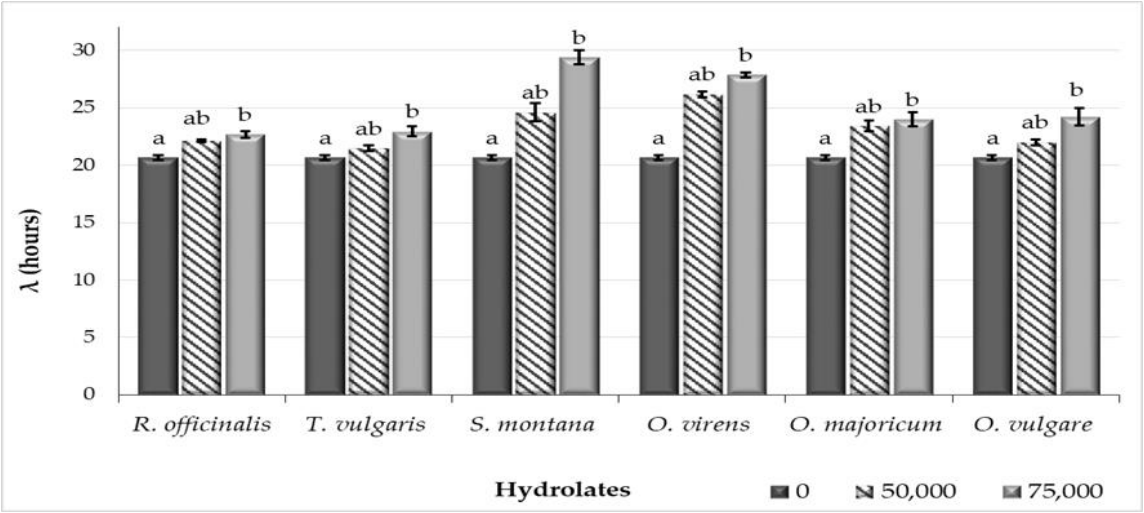

Figure 2. *Aspergillus flavus* S.44-1 lag phase (h) at different concentrations (0, 50,000 and 75,000 μg/mL) of hydrolates (*R. officinalis*, *T. vulgaris*, *S. montana*, *O. virens*, *O. majoricum* y *O. vulgare*). Each value corresponds to the mean of three replicates and the thin vertical bars represent the standard error of the data. Groups with the same letter are not significantly different ($p > 0.05$). Statistical analysis was performed independently for each compound.

Satureja montana (SM) and *O. virens* (OV) HLs had a significant effect on *A. flavus* growth rate at the maximum concentration tested (75,000 μg/mL), reducing fungal growth by 59 and 58 %, respectively (Figure 1). On the other hand, the application of *O. vulgare* HL significantly increased *A. flavus* growth (6 %) when compared to the control group.

Regarding the lag phase, all HLs had a significant effect at the maximum concentration tested, being SM and OV HLs the most effective ones with a growth delay of 9 and 8 hours, respectively (Figure 2).

Rosmarinus officinalis, *S. montana* and *O. vulgare* HLs significantly reduced AFB_1 production compared to the control group, with reductions of 61, 38 and 43 % respectively, at the maximum concentration tested. The values for AFB_2, G_1 and G_2 concentrations were low, either in the case of control group or in plates supplemented by HLs (Table 2). However, statistical analyses in some cases, showed significant differences with respect to the control. In the case of AFB_2, the treatment with *R. officinalis* showed significant differences with respect to the control group, with a reduction of 54 %. Aflatoxin G_1 concentration increased in the plates with 50,000 μg/mL of *T. vulgaris* and *S. montana*. Aflatoxin G_2 production was reduced in the presence of *T. vulgaris* and *O. majoricum* HLs at the maximum concentration tested (75,000 μg/mL) with reductions of 48 % in both cases.

Table 2. Aflatoxin concentrations (B_1, B_2, G_1, and G_2) in CYA plates supplemented with different concentrations (0, 50,000 and 75,000 μg/mL) of hydrolates (*R. officinalis, T. vulgaris, S. montana, O. virens, O. majoricum,* and *O. vulgare*). Each value is the mean of three replicates ± standard error. Groups with the same letter are not significantly different ($p > 0.05$). Statistical analysis was performed independently for each compound.

Hydrolates	μg/mL	B_1 (μg/g agar)	B_2 (μg/g agar)	G_1 (μg/g agar)	G_2 (μg/g agar)
	0	16.086 ± 2.534 ^{a}	0.319 ± 0.064 ^{a}	0.469 ± 0.043 ^{a}	0.075 ± 0.005 ^{a}
R. officinalis	50,000	8.586 ± 1.246 ^{ab}	0.159 ± 0.025 ^{ab}	0.221 ± 0.032 ^{a}	0.032 ± 0.003 ^{b}
	75,000	6.271 ± 1.129 ^{b}	0.146 ± 0.003 ^{b}	0.808 ± 0.376 ^{a}	0.060 ± 0.014 ^{ab}
	0	16.086 ± 2.534 ^{a}	0.319 ± 0.064 ^{a}	0.469 ± 0.043 ^{a}	0.075 ± 0.005 ^{a}
T. vulgaris	50,000	10.653 ± 1.492 ^{a}	0.208 ± 0.027 ^{a}	0.881 ± 0.105 ^{b}	0.045 ± 0.005 ^{ab}
	75,000	9.289 ± 0.624 ^{a}	0.221 ± 0.045 ^{a}	0.647 ± 0.076 ^{ab}	0.039 ± 0.001 ^{b}
	0	16.086 ± 2.534 ^{a}	0.319 ± 0.064 ^{a}	0.469 ± 0.043 ^{a}	0.075 ± 0.005 ^{a}
S. montana	50,000	11.757 ± 1.355 ^{ab}	0.298 ± 0.019 ^{a}	1.464 ± 0.378 ^{b}	0.063 ± 0.016 ^{a}
	75,000	9.908 ± 0.637 ^{b}	0.254 ± 0.008 ^{a}	1.082 ± 0.074 ^{ab}	0.058 ± 0.008 ^{a}
	0	16.086 ± 2.534 ^{a}	0.319 ± 0.064 ^{a}	0.469 ± 0.043 ^{ab}	0.075 ± 0.005 ^{ab}
O. virens	50,000	12.362 ± 1.817 ^{a}	0.302 ± 0.040 ^{a}	0.883 ± 0.513 ^{b}	0.111 ± 0.041 ^{b}
	75,000	12.475 ± 4.457 ^{a}	0.222 ± 0.021 ^{a}	0.348 ± 0.038 ^{a}	0.049 ± 0.004 ^{a}
	0	16.086 ± 2.534 ^{a}	0.319 ± 0.064 ^{a}	0.469 ± 0.043 ^{a}	0.075 ± 0.005 ^{a}
O.majoricum	50,000	17.018 ± 0.551 ^{a}	0.333 ± 0.013 ^{a}	0.445 ± 0.042 ^{a}	0.051 ± 0.010 ^{ab}
	75,000	12.521 ± 3.246 ^{a}	0.253 ± 0.075 ^{a}	0.301 ± 0.087 ^{a}	0.038 ± 0.008 ^{b}
	0	16.086 ± 2.534 ^{a}	0.319 ± 0.064 ^{a}	0.469 ± 0.043 ^{a}	0.075 ± 0.005 ^{a}
O. vulgare	50,000	11.239 ± 1.494 ^{ab}	0.209 ± 0.028 ^{a}	0.301 ± 0.049 ^{a}	0.036 ± 0.003 ^{b}
	75,000	9.142 ± 0.576 ^{b}	0.180 ± 0.024 ^{a}	0.643 ± 0.342 ^{a}	0.047 ± 0.005 ^{ab}

4. Discussion

As discussed earlier in this chapter, plant extracts could be applied as safe and ecofriendly preservatives to avoid post-harvest losses due to mycotoxin contamination [9,10]. Hydrolates have been long used in the cosmetics industry [11], as well as for flavouring or preserving drinks [12]. Several studies have demonstrated their antimicrobial and antioxidant activities [4-7], as well as their efficiency to control pests in during the cultivation cycle [8].

Hydrolates contain traces of EOs [2], and their chemical components include alkaloids, flavonoids, tannins, glycosides, terpenes, sterols, and lignans, which may be responsible for their powerful antimicrobial properties [4]. Hydrolates have been defined as waste products from hydro-distillation of aromatic plant extracts, but recent research works have reconsidered their use, analysing their antimicrobial and antifungal abilities [4,7]. In this work, the effect of six HLs on growth and AF production by *A. flavus* has been evaluated in vitro, comparing their efficacy with their corresponding EOs.

The chemical composition of the six EOs analysed in this chapter as well as in their HLs varies widely both in the number of active compounds and their relative concentration (Table 1). Some of the active compounds present in the EOs remain in their corresponding HLs. That is the case of SM, *R. officinalis* and *T. vulgaris* EOs, characterized by carvacrol as the major component in SM, and both eucalyptol and camphor in the other cases. On the contrary, many of the active compounds of EOs of

the three species of oregano plants tested in this work were not present in their corresponding HLs. The composition of EOs may vary widely depending on several factors such as the plant species and subspecies, geographic location or harvest time, among others [13]. Taking into account that HLs are rich in water soluble bioactive compounds, the different composition between HLs and EOs might be due to the presence of highly hydrophobic components that are not present in the water soluble fraction.

The presence of all HLs tested in this work was able to extend the lag phase, although those from SM and OV were the most effective ones. In addition, SM and OV HLs were the only ones that significantly reduced fungal growth rate. These results can be related to those reported in the present chapter, which demonstrated that these EOs (from SM and OV) were able to effectively control *A. flavus* growth and its ability to produce AFs. However, it is clear that the effect on fungal development of the EOs was by far greater than HLs and only *R. officinalis*, OV and SM HLs were able to decrease AFB_1 production. The biological activity of EOs depends on their compounds, mainly mono and sesquiterpenes, but also carbohydrates, alcohols, ethers, aldehydes and ketones [14]. During hydrodestillation process, part of the active compounds of the EOs can remain dissolved in the HLs, giving them also biological properties [3]. However, our results demonstrated that, in general, the HL chemical composition is quite different from that of theirs corresponding EOs. In regard of the most effective compounds, it is important to highlight that both SM EO and HL contain carvacrol in their composition, whereas OV HL does not present thymol as its corresponding EO. The antifungal activity of these extracts might be due to other terpenes, which can cause cell death or inhibit sporulation and germination of fungi, due to their highly lipophilic nature and low molecular weight [15]. Moreover, the involvement of other minor molecules in the enhacement of their antifungal activity might not be discarded [16]. In addition to this, several authors have attempted to correlate EOs composition and their antimicrobial activities although the action of one or two main components of the EOs seems questionable. The biological activity of EOs and their HLs is not determined by a single molecule but by a synergistic effect of the sum of all the compounds that constitute them [14,16-19].

In this study, we have demonstrated that the HLs can affect both fungal growth and, to a lesser extent, toxin production by *A. flavus*, although they are not as effective as the corresponding purified EOs. However, their proved antifungal and antitoxigenic properties may contribute to the development of HLs-based fungal control methods, providing an alternative to these waste products. As HLs are water-soluble aromatic compounds and a by-product of EO distillation, they are easy to apply and inexpensive to produce [3]. Therefore, they might be considered a valuable by-product for their spray application during cereal storage, being an alternative to synthetic chemicals.

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APPENDIX II. Optimization of the application protocol of essential oils on maize grains to prevent fungal growth and aflatoxin B_1 production by *Aspergillus flavus*.

1. Introduction

The antimicrobial activity of EOs is limited due to their high volatility, low water solubility, and susceptibility to oxidation [1,2]. To avoid these drawbacks, several authors have proposed different encapsulation techniques, being spray drying, spray cooling, extrusion, coacervation and emulsification the most used in the case of EOs [1,3-5]. All encapsulation methods depend on two components, the core (bioactive compound), and the wall material or emulsifier (that preserves the bioactive compound). The selection of the encapsulating material is a key point to optimize an application protocol for EOs and depends on several factors that should be carefully controlled before a large-scale production [1,3,4]. The application of *Satureja montana* (SM) and *Oreganum virens* (OV) essential oils (EOs) encapsulated in niosomes (lipid-based systems) to control growth and aflatoxin (AF) production by *Aspergillus flavus* on maize grains was successfully described in this chapter.

This appendix shows the preliminary EO application trials, which led to the selection of the niosome-based method for encapsulation of SM and OV EOs to effectively control AF contamination during maize storage. The effectiveness of the EO applied without encapsulation (direct application, spraying and volatilization of EOs) was compared to that achieved using the EO encapsulated using four different methods (niosomes, Arabic gum, alginate, and gelatine encapsulation).

2. Materials and Methods

The optimization of the best protocol to apply the EOs in maize were carried out using thyme essential oil (TEO) commercially available from Biogran S.L. (Madrid, Spain).

These trials were conducted in Petri dishes of 90 mm filled with crystalized potassium sulphate (Figure 1). In the center of it, a 50 mm Petri dish containing 7 g of inoculated maize with *A. flavus* (10^4 spores/g) was placed. The methodology was described in this chapter and a detailed protocol was included in section 2.3.2 "Effect of niosome-encapsulated essential oils on fungal growth and aflatoxin production on maize grains". The *A. flavus* strain evaluated was the same used in the chapter 3, A7, which produces AFB_1, B_2, G_1 and G_2 and was isolated from Spanish corn in our group.

Figure 1. Corn inoculated with *A. flavus* (A7) on Petri dishes with potassium sulfate crystals and an essential oils container.

The inoculated corn grains were subsequently treated with TEO, applied using different methods, as described below (sections 2.1 and 2.2). All trials were tested in triplicate, the plates were incubated at 28 °C, during 7, 9 and 28 days, depending on the trial.

After the incubation period, a sample of 3.5 g was taken from each treatment to evaluate fungal growth by determination of colony forming units (CFUs) per gram, and the rest of the sample (3.5 g) was used for AFB_1 evaluation. The detailed protocols were described in this chapter (section 2.3.2.1. "Small-scale assays" and section 2.4.2. "Detection of mycotoxins by thin layer chromatography"). The evaluation of AFB_1 production was carried out following the criteria represented in Table 1. The thickness and intensity of the spots were related to different levels of AFB_1 concentration.

Table 1. Evaluation of AFB_1 production by thin layer chromatography.

+++		High production
++		Medium production
+		Low production
-		Not detectable

2.1. Effect of non-immobilized thyme essential oil on growth and aflatoxin production by **Aspergillus flavus** *in maize grains*

After inoculation of the grains with *A. flavus*, three different methods of application of non-immobilized TEO were performed: direct spread on the grains, by spraying or by volatilization.

The TEO was diluted in polyethylene glycol 400 (PEG (Acros, Geel, Belgium)) to obtain a stock solution at 2,100 μg/mL. The stock solution was filtered (pore size 0.2 μm (Fisherbrand, Shanghai, China)) and 1 mL was added using the corresponding application method to the inoculated corn grains, to obtain a final concentration of 300 μg of TEO per gram of corn.

The direct application was made by spreading TEO on the inoculated corn grains, whereas the spraying application was made with an airbrush (Parkside, Germany), with an air pressure of 2.6 bar and a spraying distance of approximately 20 cm. On the other hand, a third application was made by introducing the TEO in a small container and letting the EO volatilize during the incubation time (Figure 1). Control assays were also included using sterile water instead of TEO. Incubation was performed at 28 °C and the effect of TEO on fungal development and AFB_1 accumulation on maize grains was evaluated after 9 and 28 days. In all cases, 3 replicates per treatment were carried out.

2.2. *Effect of encapsulated thyme essential oil on growth and aflatoxin production of* **Aspergillus flavus** *in maize grains*

Four encapsulation methods (Arabic gum, gelatin, alginate and niosomes) were evaluated in this study and encapsulated TEO was applied on corn grains after inoculation with *A. flavus* A7. The TEO was diluted in PEG-400 and added to the different solutions to obtain final concentrations of 2,100 μg/mL. The same amount of water was included in the control instead of TEO. The protocols used to encapsulate TEO are described below.

a) Encapsulation in Arabic gum or gelatine. Both Arabic gum and gelatine solutions were prepared at 4 % in distilled water, following the protocol described by Herranz-Gómez [6]. Subsequently, TEO was added to each solution. Both solutions, Arabic gum and gelatine were stirred for 1 minute and incubated at 50 °C in a water bath (Bunsen S.A, Spain) until homogenisation. Subsequently, the solutions were stirred 2 minutes and their pH was adjusted to 4 using 37 % HCl. Once the pH was adjusted, the solutions were kept at 8 °C for 1 hour. Then, their pH was readjusted to 9 (NaOH). Subsequently, 3.5 g of 50 % glutaraldehyde (Alfa Aesar, Spain) was added and stirred for 5 minutes.

b) Encapsulation in alginate. In this case, TEO was primarly added to a sodium alginate solution at 2 % in distilled water. Subsequently, the solution were stirred and heated in a heating plate (Bunsen S.A., Spain), until homogenisation. The solution was kept at 25 °C for 45 minutes and then, it was dripped into calcium chloride solution at 2 %. Two sizes of alginate droplets (diameter 0.2 and 0.3 cm) were tested.

c) Encapsulation in niosomes. The microencapsulation in niosomes was carried out following the manufacturer's instructions (Nanovex, Biotechnologies S.L., Spain). Preheated water (55 °C) and TEO were added in vials containing the niosome formulation. The vials were stirred and incubated at 55-65 °C for 20 minutes in a water bath (Bunsen S.A., Spain), shaking every 5 minutes. Subsequently, vials were immersed for 15 minutes in an ultrasonic sonicator (P Selecta, Spain), in order to reduce the size of the vesicles.

In all cases, 1 mL or 1 g of encapsulated TEO were applied to the plate containing the 7 g of inoculated corn (Figure 2), obtaining a final concentration of 300 μg/g. Incubation was performed at 28 °C and the effect of TEO encapsulation on fungal growth and AFB_1 accumulation on the grains was evaluated as described above after a 7 day-incubation. In all cases, 3 replicates per treatment were carried out.

(a) (b) (c)

Figure 2. Corn grains inoculated with *A. flavus* A7 and treated with TEO encapsulated in Arabic gum (a), alginate (b) and niosomes (c).

2.3. Statistical analysis

The effect of TEO on fungal growth was evaluated using analysis of variance (ANOVA). Statistical analysis was performed using the statistical software StatsGraphics Centurion XVII V.17.2.04 (Statpoint Technologies Inc., Warrenton, VA, USA). The Shapiro–Wilk and Levene tests were used to check normality and homoscedasticity, respectively.

3. Results

3.1. Effect of non-immobilized thyme essential oil on Aspergillus flavus *growth and aflatoxin production in maize grains*

The presence of TEO applied to the inoculated corn grains both directly and sprayed, significantly reduced *A. flavus* A7 growth compared to the control, after 9 and 28 days of incubation (Table 2). On the other hand, volatilized TEO did not significantly reduced fungal growth on corn grains at any incubation time.

High levels of AFB_1 produced by *A. flavus* were detected in control groups (after 9 and 28 days of incubation) as well as in corn grains treated with volatilized TEO (Table 2). The AFB_1 detected in the grains after 9 days of incubation was low when TEO was sprayed, and the concentration was under the detection limits in the case it was applied directly. When the incubation time was increased to 28 days, similar results were observed, except for direct TEO treatment, in which high concentrations of AFB_1 were detected.

Table 2. Effect of thyme essential oil effect applied by direct contact, volatilized and sprayed on *Aspergillus flavus* growth and aflatoxin B_1 (AFB_1) production, incubated for 9 and 28 days. Each value is the mean ± standard error of three replicates. Groups with the same letter are not significantly different ($p > 0.05$). Statistical analysis was performed for each incubation time.

INCUBATION TIME (days)	APLICATION	CFU/g	AFB_1
9	CONTROL	$2.10x10^8 \pm 6.68x10^7$ a	+++
	DIRECT	$1.06x10^6 \pm 1.16x10^6$ b	-
	VOLATILIZED	$1.40x10^9 \pm 7.48x10^7$ a	+++
	SPRAYED	$3.76x10^5 \pm 1.74x10^5$ b	+
28	CONTROL	$1.40x10^9 \pm 2.82x10^8$ a	+++
	DIRECT	$2.53x10^8 \pm 5.79x10^7$ b	++
	VOLATILIZED	$6.66x10^8 \pm 2.05x10^8$ ab	+++
	SPRAYED	$5.36x10^8 \pm 3.25x10^7$ c	+

CFU (colony forming units). High (+++), medium (++), low (+) and not detectable (-) AFB_1 concentration.

3.2. Effect of encapsulated thyme essential oil on the growth of **Aspergillus flavus** *in maize grains*

Table 3 shows the results regarding fungal growth and AFB_1 detection on inoculated maize grains treated by encapsulated TEO. Only the application of niosome-encapsulated TEO was able to significantly reduce *A. flavus* growth with respect to the corresponding control after 7 days of incubation. Similarly, the application of niosome-encapsulated TEO was the only treatement which were able to affect AFB_1 concentration. In the case of both Arabic gum and gelatine encapsulation, AFB_1 was not detected even in the control assays, so it is assumed that the encapsulating material or the encapsulation process may be affecting AF detection.

Table 3. Effect of thyme essential oil encapsulated by different methods (Arabic gum, gelatine, alginate and niosomes) on *Aspergillus flavus* growth and aflatoxin B_1 (AFB_1) concentration detected on the grains. Each value is the mean ± standard error of three replications. Groups with the same letter are not significantly different ($p > 0.05$). Statistical analysis was performed for each treatment.

TREATMENT	APLICATION	CFU/g	AFB_1
ARABIC GUM	CONTROL	$1.70x10^4 \pm 4.96x10^3$ a	-
	TEO	$1.33x10^4 \pm 1.70x10^3$ a	-
GELATINE	CONTROL	$2.06x10^4 \pm 1.40x10^4$ a	-
	TEO	$2.80x10^4 \pm 5.71x10^3$ a	-
ALGINATE 0.2 cm	CONTROL	$1.43x10^8 \pm 5.43x10^7$ a	+++
	TEO	$2.47x10^8 \pm 1.66x10^8$ a	+++
ALGINATE 0.3 cm	CONTROL	$1.43x10^8 \pm 5.43x10^7$ a	+++
	TEO	$2.00x10^8 \pm 9.09x10^7$ a	+++
NIOSOMES	CONTROL	$1.12x10^7 \pm 1.69x10^5$ a	+++
	TEO	$9.10x10^5 \pm 2.16x10^4$ b	+

CFU (colony forming units). High (+++), medium (++), low (+) and not detectable AFB1 production (-).

4. Discussion

As mentioned in this chapter, many EOs have been described as potent antifungal compounds. Alzate et al. (2009) attributed this activity to their volatile components [7]. However, the results obtained in this study showed that the effective control of *A. flavus* growth and AFB_1 production needs direct contact between the TEO and the inoculated maize grains. Moreover, the results obtained suggest that TEO antifungal effect is lost over time, probably due to the volatilization of its active components. In order to address these drawbacks, several authors consider the use of encapsulation techniques as a good option to reduce the loss of active principles, to improve their bioavailability, and to protect the EOs against environmental factors, especially temperature, light, and oxygen [2,3,5,8]. In this study, different encapsulation techniques have been evaluated in order to select to most appropriate method to maximize the antifungal and antitoxigenic effect of EOs.

Wu et al. (2015) demonstrated that alginate and Arabic gum were effective encapsulating material because they produce a sustained release of the EO and improve its antimicrobial properties [9]. However, the results obtained in this work showed the inability of alginate, and Arabic gum to encapsulate TEO properly to be used to inhibit *A. flavus* growth. Soliman et al. (2013) reported a reduction in the evaporation rate of clove, thyme and cinnamon EOs encapsulated in 2 % alginate microspheres sprayed into 0.5 % calcium chloride solution, and also an increased in their antifungal activity [10]. However, in this case, TEO encapsulated in both size alginate beads (0.2 and 0.3 cm) did reduce neither *A. flavus* growth nor AFB_1-production. This may be due to the high concentration of calcium chloride (2 %), which could have trapped TEO inside the spheres, preventing its release during the incubation times tested in this study. According to Manjana et al. (2009) an increase in calcium chloride concentration supposes an improved loading capacity of the alginate microspheres but also an increase in EO entrapment due to the thick wall of the alginate microspheres [11]. This fact might be influencing the release of the active compounds which would need longer times to cross the particle wall and, therefore, the EO could drastically loss its effect [3,12,13].

Girardi et al. (2016, 2017) prepared EOs microcapsules using the complex coacervation technique with gelatin and Arabic gum and their application suppose a significant reduction the growth of *Penicillium* sp. and *Aspergillus* sp. in peanuts [14,15]. These results differ from those reported in this work in which TEO encapsulated in Arabic gum and gelatin had no effect on *A. flavus* growth. Several factors can influence the efficiency of EOs encapsulation by the coacervation technique with gelatin and Arabic gum, specially pH and temperature [1]. These parameters determine the size of the particles, but can also contribute to the degradation of some encapsulated active compounds [16]. The results obtained in this work showed that even in control assays *A. flavus* drastically reduce its growth compared to the rest of control treatments. This fact can be due to the necessity to use glutaraldehyde during encapsulation process, since it has been reported as a toxic product to *A. flavus* [17]. Similar results were obtained in the case of AFB_1 production that seemed to be affected by the encapsulation process. Several authors have reported the use of hydrocolloids such as

gelatin, cyclodextrins and other carbohydrates to remove mycotoxins in food matrices [18,19]. The gelatin or the Arabic gum used for encapsulation might be able to detoxify this toxin, although more assays are necessary to reach a clear conclusion.

Among the four EO encapsulation methods tested in this work, the only case in which the TEO was able to reduce growth and AFB_1 production by *A. flavus* in inoculated corn grains was in the case of niosome-encapsulated EO application. Liolios et al. (2009) investigated different parameters, such as the antioxidant activity or the antimicrobial activity of thymol and carvacrol, before and after their encapsulation in lipid-based systems, such as niosomes [20]. Their results revealed a reduction in the oxidation of active compounds, and the antimicrobial activities of these bioactive compounds considerably increased after their encapsulation in niosomes. Wen et al. (2011) also reported that these encapsulation methods protect from heat, preventing thermo-degradation, and provide slow release of the encapsulated active compounds [21].

As already discussed in this chapter, niosomes-based systems are non-toxic, very stable and easily stored and handled, which are important advantages for their application in the food industry. Essential oils are a complex volatile mix of bioactive compounds with antifungal and antitoxigenic properties and, therefore, niosomes-based encapsulation systems may be appropriate for their application in food matrices, such as cereals. Niosomes will protect the EOs from adverse conditions such as temperature, pH, or oxidation), as well as provide a controlled release of the active compounds. Moreover, niosomes have the advantage of being commercially available, so their formulation is well performed to prevent interassay differences. Besides, the size of niosomes (0.27-0.3 μm) is smaller than the vesicles of the Arabic gum or gelatine vesicles (86-300 μm) and alginate (0.2-0.3 cm) [3], so that their application could be easily carried out using spray systems. The niosome vesicles can be dispersed using an aqueous phase and can be directly applied in a non-aqueous solid matrix [as reported in this chapter], such as cereal grains. The vesicles will control the release of the EOs, effectively reducing fungal growth and mycotoxin production in the cereals over the storage period.

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CHAPTER 4

Assessment of the effect of *Satureja montana* and *Origanum virens* essential oils on *Aspergillus flavus* growth and aflatoxin production at different water activities.

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Abstract

Aflatoxin contamination of foodstuffs poses a serious risk to food security, and it is essential to search for new control methods to prevent these toxins entering the food chain. Several essential oils are able to reduce the growth and mycotoxin biosynthesis of toxigenic species, although their efficiency is strongly influenced by the environmental conditions. In this work, the effectiveness of *Satureja montana* and *Origanum virens* essential oils to control *Aspergillus flavus* growth was evaluated under three water activity levels (0.94, 0.96 and 0.98 a_w) using a Bioscreen C, a rapid in vitro spectrophotometric technique. The aflatoxin concentrations at all conditions tested were determined by HPLC-FLD. *Aspergillus flavus* growth was delayed by both essential oil treatments. However, only *S. montana* essential oil was able to significantly affect aflatoxin production, although the inhibition percentages widely differed among water activities. The most significant reduction was observed at 0.96 a_w, which is coincident with the conditions in which *A. flavus* reached the highest levels of aflatoxin production. On the contrary, the treatment with *S. montana* essential oil was not effective in significantly reducing aflatoxin production at 0.94 a_w. Therefore, it is important to study the interaction of the new control compounds with environmental factors before their application in food matrices, and in vitro ecophysiological studies are a good option since they provide accurate and rapid results.

Keywords

Aflatoxin; bioscreen; preservatives; essential oils; food security

Key Contribution

The effect of natural compounds on the growth of toxigenic species and on their ability to produce mycotoxins widely varied regarding the doses and the environmental conditions in which they were applied. Therefore, it is important to develop accurate and rapid methods to easily evaluate a combination of different parameters.

1. Introduction

Mycotoxins are fungal secondary metabolites with adverse effects on human and animal health. To date, more than 400 different molecules, produced by several types of fungi, have been characterized [1]. Because of their high toxicity, aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, and AFG_2) are the most important, and are produced by the species of *Aspergillus* section *Flavi*, mainly *Aspergillus flavus* [2]. These toxins can contaminate a wide range of agricultural commodities, either in the field or during storage, and they are considered ubiquitous contaminants of the food supply throughout the developing world [3]. AFB_1 has been described as the most toxic naturally occurring human carcinogen and as the cause of hepatocellular carcinoma in humans and animals [3,4]. The International Agency for Research on Cancer (IARC) has classified the "naturally occurring mixes of aflatoxins" as a Group 1 human carcinogens [5].

Aflatoxin (AF) contamination of feed and food products poses a serious risk to food security and leads to important economic losses due to the impossibility to market contaminated products as well as veterinary and health costs. Most countries have established maximum levels of these contaminants allowed in food products [5]. One of the main problems is that mycotoxins are extremely stable compounds. They are heat-resistant, with melting temperatures above 250 °C, and tolerate a wide pH range, from 3 to 10 [6,7]. Furthermore, mycotoxins have nondetectable sensory characteristics and they do not change the organoleptic properties of food products. Thus, once mycotoxins are present in raw ingredients, they are very difficult to eliminate. Therefore, preventing contamination is the best approach and thus it is essential to establish adequate control methods to prevent AFs from entering the food chain [8]. The best strategy to prevent their presence in agrifood products is to completely avoid fungal growth. However, this might be complicated due to the way food and feed materials are harvested, stored and processed. Moreover, the presence of the fungus is not always associated with the presence of mycotoxins, as the ecological conditions for mycotoxin production are narrower than for fungal growth. Thus, it becomes more important to look for methods that not only focus on controlling fungal growth in food matrices, but also on preventing the synthesis of mycotoxins [6,9].

Many factors influence *A. flavus* growth and AF production in food products, including nutritional composition, temperature, pH, water activity (a_w), atmospheric composition, and storage times, as well as the presence and concentration of preservatives [10]. The application of food preservatives to control mycotoxin-producing fungi is effective. However, consumers are now demanding safer and more ecofriendly products free from chemicals. In this context, natural plant extracts are considered to be good alternatives [11].

Essential oils (EOs) are aromatic extracts obtained mostly from plant material and have demonstrated strong antimicrobial, antitoxigenic and food preservative properties as well as low toxicity towards animals and humans [12]. They are allowed in food products, have less environmental impact and, therefore, a wider public acceptance [13]. These natural plant extracts are recognized as safe on the GRAS (Generally Recognized As Safe) list, and are used in various sectors, such as agriculture

(plant fortifiers, biostimulants, pesticides, postharvest or herbicides), food industry (preservatives or flavorings) and pharmaceutics (aroma compounds or functional ingredients) [13]. In addition, their use is approved for ecological agriculture [14]. Several EOs have been reported to reduce not only the growth of toxigenic fungal species but also to interfere in mycotoxin biosynthesis to some extent [15,16]. *Satureja montana* (SM) and *Origanum virens* (OV) EOs are highly rich in carvacrol and thymol, respectively, which are responsible for their antifungal properties [17].

It is well known that controlling the dose of preservatives is crucial, since suboptimal concentrations could lead to stimulation of both growth and toxin accumulation [18]. The efficacy of EOs is also influenced by the environmental conditions, mainly a_w and temperature [19]. Therefore, it is important to unravel the interactions between these environmental factors and antifungal compounds. The study of these interactions required laborious in vitro ecophysiological studies to evaluate fungal growth and mycotoxin production, which requires the use of a lot of material and the planning of long-term experiments. The use of Bioscreen-C Microbiological Growth Analyzer for mycological studies is a quick method to study the effects of multiple factors on mold growth [10,20,21].

The aim of this work was to evaluate the effect of two EOs extracted from SM and OV on the early growth and ability to produce AFs by two *A. flavus* strains (A7 and A10) at several EO concentrations (0, 350, 700, and 1000 μg/mL) and three a_w conditions (0.94, 0.96, and 0.98).

2. Materials and Methods

2.1. Microorganism and essential oils

2.1.1. Fungal strains

Two aflatoxin-producing strains of *A. flavus* were used (A7 and A10). They were isolated from maize and oats in different works performed in our laboratory. The correct identification of these isolates was confirmed using a species-specific PCR protocol [22]. The strains were selected due to their ability to produce aflatoxins (AFs). The strain A10 was classified as a high toxin-producing isolate (5.90 ng/μL AFB_1 + 0.43 ng/μL AFB_2), whereas the strain A7 was able to produce low levels of AFs (0.33 ng/μL AFB_1 + 0.03 ng/μL AFB_2).

The strains were maintained by regular subculturing on potato dextrose agar medium (PDA (Pronadisa, Madrid, Spain)) at 25 ± 1 °C for 5 days in the dark, and stored as a spore suspension in 15 % glycerol (Panreac, Madrid, Spain) at −80 °C until required.

2.1.2. Essential oils of plant

The essential oils (EOs) tested from *Satureja montana* L. (SM) and *Origanum virens* Hoffmanns and Link (OV) were provided by The Agricultural Research Centre

of Albaladejito (Cuenca, Spain). Extraction was previously described by García-Díaz et al. (2019) [28]. Briefly, each plant species was extracted by hydrodistillation of the dried aerial parts of aromatic plants, following the methodology proposed by the European Pharmacopoeia in a Clevenger-type apparatus for 2 hours. The chromatograms are shown in Appendix B.

These compounds were filtered with sterile 0.22 μm pore size filters (Fisher Scientific, Madrid, Spain) and stored at −20 °C in amber glass vials (Thermo Scientific, Madrid, Spain), until required.

2.2. *Experimental design*

Semisolid YES (Yeast Extract Sucrose) medium (20 g/L of yeast extract, 150 g/L of sucrose, 0.5 g/L of magnesium sulfate and 0.5 g/L of agar [20]) at different water activities (a_w) (0.94, 0.96, and 0.98) was spiked with different concentrations of the EOs. The a_w of the YES medium was modified by substituting water with glycerol [23].

The essentials oils of SM and OV were diluted in 5 mL of YES media to obtain final concentrations of 350, 700, and 1000 μg/mL. The control medium was supplemented by the same volume of water instead of EO.

The initial spore suspensions of each strain were prepared in sterile saline solution (9 g/L sodium chloride (Merck, Darmstadt, Germany)). After homogenizing, the spore concentrations of the solutions were measured using a Thoma counting chamber (Marienfeld, Lauda-Königshofen, Germany) and then adjusted with a sterile solution to a final concentration of 10^7 spores per mL.

Every medium was inoculated with 50 μL of a 10^7 spores/mL suspension of the corresponding strain. The resulting final concentration was 10^5 spore/mL in the YES medium for each strain, EO concentration and a_w. A total of 48 conditions were evaluated. Ten replicates per treatment were carried out.

Three hundred μL of inoculated media, as well as noninoculated controls, were placed in 100-well honeycomb plates and incubated at 25 ± 1 °C for 7 days in the Bioscreen C Microbiological Growth Analyser (Labsystems, Helsinki, Finland).

The optical density (O.D) was automatically recorded every 30 minutes using a 600 nm filter over 7 days (10,080 minutes). The data were recorded using the software Easy Bioscreen Experiment (EZExperiment) provided by the manufacturer and then exported to a Microsoft Excel Professional 2010 (Microsoft Corporation, Washington, USA) datasheet for further analyses.

2.3. *Aflatoxin assessment*

For each treatment and condition, 3 replicates corresponding to the content of 3 wells were transferred to 2 mL Eppendorf tubes. AF extraction was carried out with 0.8 mL of chloroform (Merck, Darmstadt, Germany), by vigorous shaking for 60 minutes. The mix was then centrifuged for 5 minutes at 5000 rpm (Centrifuge 5417 R (Eppendorf, Stevenage, UK)). The aqueous phase was decanted, and the chloroform

phase was transferred to a new tube. The samples were evaporated to dryness in a miVac vacuum centrifuge (SP Scientific, Suffolk, United Kingdom), and the residues were redissolved in 500 μL methanol/water (50:50; *v*/*v*). The samples were filtered using a nylon syringe filter, 0.22 μm pore size (Minisart®, Sartorius Stedim, Germany), and were transferred into HPLC-FLD vials and stored at −20 °C until analysis.

The samples were analyzed by an HPLC-FLD detector (Agilent1200 series HPLC, Agilent, Cheadle, UK), coupled to a UVE photochemical derivatizer (LCTech, Obertaufkirchen, Germany). The FLD detector excitation and emission wavelengths were 330 and 460 nm, respectively. Chromatographic separations were performed on a C_{18} column ZORBAX-Eclipse Plus (4.6 × 150 cm, 3.5 μm (Agilent, Cheadle, UK)). Methanol/water/acetonitrile (30:60:10; *v*/*v*/*v*) was used as the mobile phase at a flow rate of 1 mL/min. AFG_2, AFG_1, AFB_2 and AFB_1 were eluted at 6.5, 7.6, 8.7 and 10.4 min, respectively. The signals were processed by Agilent Chem-Station software (Agilent Technologies, Palo Alto, CA, USA). AFs were quantified on the basis of the HPLC fluorimetric response compared to a range of mycotoxin standards supplied by Romer Labs (Romer Labs, Runcorn, UK). The limit of detection (LOD) of the analysis was 0.52 ng for AFB_1 and AFG_1, and 0.06 ng for AFB_2 and AFG_2, based on a signal to noise ratio of 3:1.

2.4. Data analysis

The raw datasets obtained from the Bioscreen C were subjected to two further steps before analysis. First of all, the average of the first 5 measurements (180 minutes) for each well was calculated, and the average was subtracted from all subsequent measurements in order to correct the different signal backgrounds. Subsequently, the time to detection (TTD) for 0.2 nm of O.D was obtained using a Microsoft Excel template (kindly provided by Dr. R. Lambert), which used linear interpolation between successive O.D readings [24].

Once the TTDs were obtained, analysis of variance (ANOVA) was performed using the different concentrations of EOs (0, 350, 700 and 1000 μg/mL) and a_w (0.94, 0.96 and 0.98) as independent variables to evaluate the effect of SM and OV essential oils on the fungal growth of A7 and A10 strains of *A. flavus*. In all cases, statistical analysis was performed independently for each EO and isolate. Because of the lack of normality of the AF production datasets, ANOVA analysis was performed using a log-transformed dataset. The mean comparisons for each independent variable (EO concentration and a_w) were done using Tukey's HSD. The statistical package JMP 8 (SAS Institute Inc., 2008; Cary NC, USA) was used in the analysis.

3. Results

3.1. *Effect of* Satureja montana *and* Origanum virens *essential oils under different water activities on* Aspergillus flavus *growth*

The growth curves obtained using Bioscreen-C for the two strains of *A. flavus* (A10 and A7), in Yeast Extract Sucrose (YES) medium supplemented by different concentrations (0, 350, 700, and 1000 μg/mL) of *Satureja montana* (SM) and *Origanum virens* (OV) essential oils (EOs), under the three water activities tested (0.94, 0.96, and 0.98 a_w), are shown in Appendix A. As an example, Figure 1 shows the growth curve of *A. flavus* A7 strain at 0.94 a_w with different OV EO concentrations. The growth curves represent the optical density (O.D) units at 600 nm over time.

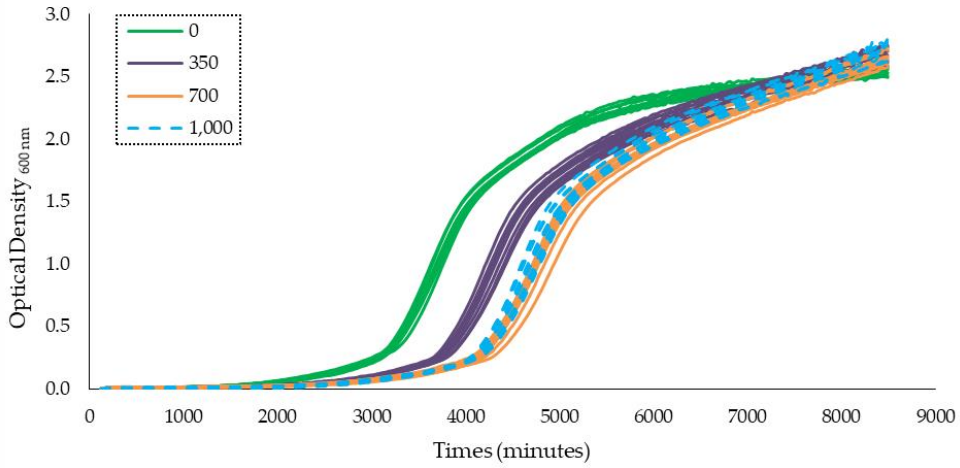


Figure 1. Growth curve obtained using the Bioscreen C analyzer representing optical density at 600 nm for 6 days for *A. flavus* A7 at 0.94 a_w. Ten replicates for each concentration (0, 350, 700, and 1000 μg/mL of OV EO) tested are represented. Concentrations of essential oils are represented in the legend.

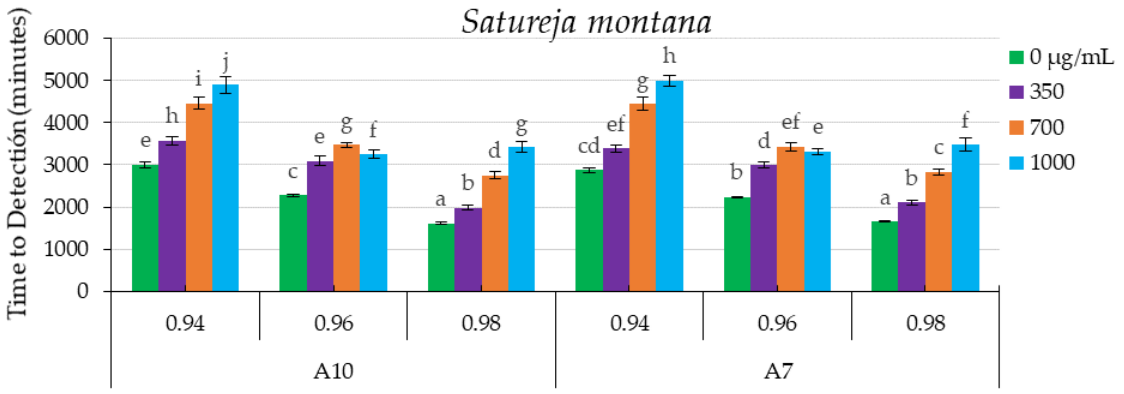
In this study, comparisons between treatments were done using the time to detection (TTD). TTD is described as the necessary time for fungal growth to reach a specific O.D level with a treatment. Medina et al. (2012) also described a direct relationship between O.D and *A. flavus* biomass [20]. In this work, the TTD was calculated using an O.D at 600 nm = 0.2.

Figure 2 shows the TTD for all the combinations of EO concentrations and a_w for the two *A. flavus* strains tested.

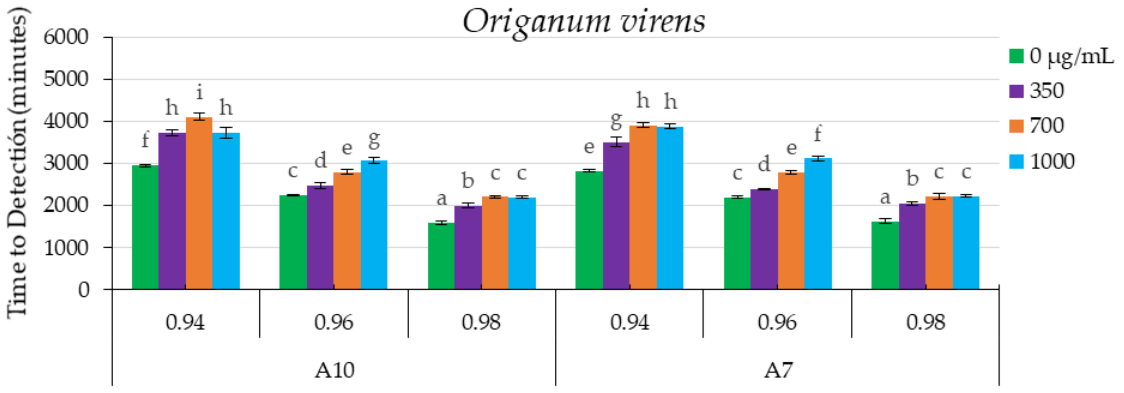
The statistical analyses regarding the influence of SM EO treatment on the growth of both strains (Figure 2a) showed a significant effect of EO concentrations ($p < 0.0001$), and a_w levels ($p < 0.0001$). The interaction between both factors (a_w and EO concentration) was also statistically significant ($p < 0.0001$). The highest antifungal properties, related to the less favorable conditions for growth (highest TTD), were obtained with 1000 μg/mL of SM EO and 0.94 a_w, with approximate TTD values of 4900 and 5000 minutes in the cases of A7 and A10 strains, respectively. There was a direct relationship between the highest SM EO concentrations and increases in TTD, showing delayed fungal growth. Interestingly, at 0.96 a_w and for both *A. flavus* strains, more

inhibition was observed at 700 µg/mL than at 1000 µg/mL. In general, the treatment with SM EO retarded fungal growth in relation to the corresponding control at all a_w levels tested.

The statistical analyses showed that, for OV EO, the growth of both strains tested (Figure 2b) was significantly affected by the EO concentrations ($p < 0.0001$) and a_w levels ($p < 0.0001$). As previously shown, the interactions between both factors were also statistically significant ($p < 0.0001$). The highest antifungal effect was observed at 0.94 a_w and 700 µg/mL, with values of approximately 4000 minutes for both isolates of *A. flavus*. For the A7 strain, there were no significant differences between the two higher doses tested (700 and 1000 µg/mL) of OV EO at 0.94 and 0.98 a_w. In the case of the A10 strain, the maximum delay in fungal growth was obtained at 0.94 a_w and 700 µg/mL. In all a_w conditions tested, the application of OV EO delayed fungal growth with respect to the corresponding control.



(a)



(b)

Figure 2. Time to detection (TTD, minutes) at 0.2 nm of Optical Density (O.D) of fungal growth of two *A. flavus* strains (A10 and A7) under different water activity levels (0.98, 0.96 and 0.94 a_w) at different concentrations (0, 350, 700 and 1000 µg/mL) of *Satureja montana* (**a**) and *Origanum virens* (**b**) essential oils. Values are the means of 10 replicates ± standard errors. Means with a common letter are not significantly different ($p > 0.05$). Concentrations of essential oils are represented in the legend. In all cases statistical analysis was performed independently for each essential oil (EO) and isolate.

In order to further study the effect of different concentrations of EOs and their interaction with environmental factors in Figure 3, we represented the rate to detection (RTD, 1/TTD) at 0.2 nm calculated in all conditions tested and normalized by the RTD_0, which corresponds to the control without EO treatment (RTD/RTD_0). If the effectiveness of the EOs was the same under the different environmental conditions, in these graphs, the lines should be superposed to each other. This also allows for the comparison of the efficacy between different antifungals.

In the specific case of SM EO shown in Figure 3a,b, it can be observed that wetter conditions (0.98 a_w) will allow higher control under the highest concentrations in comparison with the other dryer conditions tested. For OV EO, it can be observed in Figure 3c,d that there is a clear interaction at 0.96 a_w where the antifungal effect is decreased.

For both *A. flavus* isolates, the presence of SM EO showed higher antifungal activity compared to OV EO treatment at all the a_w levels tested.

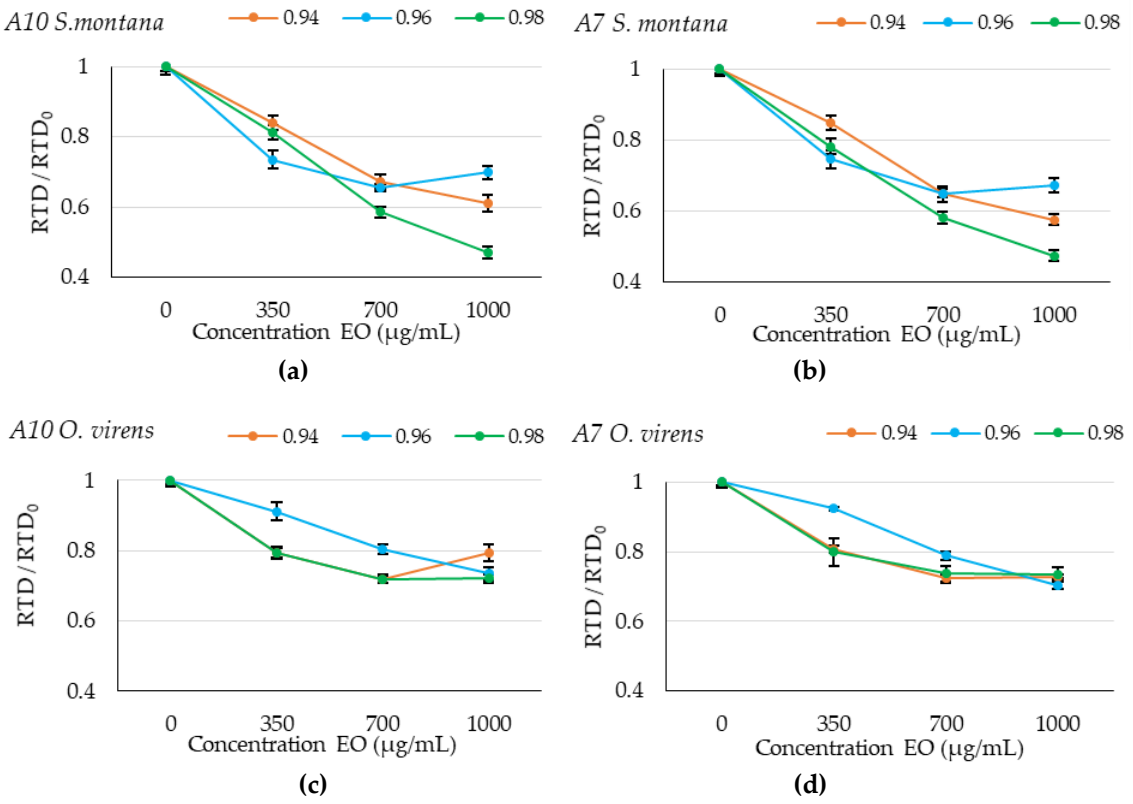


Figure 3. Graphical representation of relative rate to detection (RTD) (RTD/RTD_0) at different concentrations (0, 350, 700 and 1000 µg/mL) of *Satureja montana* (SM) and *Origanum virens* (OV) essential oils; (**a**) A10 strain with SM essential oil, (**b**) A7 with SM essential oil, (**c**) A10 strain with OV essential oil and (**d**) A7 strain with OV essential oil. The different a_w levels studied (0.94, 0.96 and 0.96) are represented in the legend. Data represent the average of the relative RTD of 10 replicates.

3.2. Effectiveness of Satureja montana *and* Origanum virens *essential oils at different water activity levels in reducing aflatoxin production*

The amount of aflatoxin B_1 and B_2 (AFB_1 and AFB_2) produced after 7 days of incubation at three a_w after the treatments with SM and OV EOs was determined, and results are shown in Table 1.

The statistical analyses regarding AFB_1 and AFB_2 produced by *A. flavus* A10 strain in the presence of SM EO showed significant differences among concentrations ($p < 0.0001$), a_w ($p < 0.0001$) and their interaction ($p < 0.0001$). Comparing between strains, the A10 isolate was able to produce higher levels of AFB_1 and AFB_2. Significant reductions were achieved for both toxins when SM EO was used at 0.96 and 0.98 a_w, with percentages of 85 % and 94 %, respectively, in AFB_1 concentrations of 1000 μg/mL of SM EO. For AFB_2, 90 % and 94 % reductions were observed at the same dose of SM EO. It is important to highlight the reduction in AF production found at 0.96 a_w. *Aspergillus flavus* A10 reached very high levels of production in control assays and more than 84 % of reduction was obtained even at the low dose of SM EO (350 μg/mL) tested.

The treatment with OV EO had a lower effect on AF production. The statistical analyses of AFB_1 for the A10 strain showed significant differences between a_w ($p < 0.0001$) but no effect on EO concentration ($p = 0.3140$) or the interaction of both factors ($p = 0.3323$). In the case of AFB_2, there were significant differences among the a_w levels ($p < 0.0001$) and EO concentrations ($p = 0.050$), whereas the interaction between factors was not significant.

Aspergillus flavus A7 produced lower levels of AFB_1 and AFB_2 than A10. In most cases, the levels were below the detection limits. The statistical analyses regarding AFB_1 and AFB_2 produced in the presence of SM EO showed significant differences among the concentrations ($p < 0.0001$) and a_w levels ($p < 0.0001$), as well as a significant interaction between both factors ($p < 0.0001$). The OV EO treatment showed significant differences among a_w levels ($p < 0.0001$) and EO concentrations ($p_{B1} < 0.0001$ and $p_{B2} = 0.0040$), as well as the interaction of factors ($p_{B1} = 0.0003$ and $p_{B2} = 0.0008$).

Table 1. Aflatoxin concentrations (B_1 and B_2) produced by *A. flavus* isolates (A10 and A7) in the presence of different concentrations (0, 350, 700 and 1000 μg / mL) of *Satureja montana* (SM) and *Origanum virens* (OV) essential oils (EOs), under different water activity levels (0.98, 0.96 and 0.94 a_w). Values are the means of 3 replicates ± standard errors. Means with a common letter are not significantly different ($p > 0.05$). In all cases statistical analysis was performed independently for each EO and isolate. N.D: Not detected (values below detection limits).

E.O	a_w	μg/mL	*A. flavus* A 10		*A. flavus* A 7	
			B_1 (μg/g agar)	B_2 (μg/g agar)	B_1 (μg/g agar)	B_2 (μg/g agar)
SM	0.94	0	751 ± 79 ^{ab}	28 ± 2 ^{a}	N.D ^{a}	N.D ^{a}
		350	786 ± 762 ^{ab}	32 ± 34 ^{a}	N.D ^{a}	N.D ^{a}
		700	572 ± 143 ^{a}	28 ± 7 ^{a}	22 ± 7 ^{a}	N.D ^{a}
		1000	404 ± 35 ^{a}	27 ± 2 ^{a}	N.D ^{a}	N.D ^{a}
	0.96	0	58,235 ± 3061 ^{d}	1856 ± 114 ^{d}	56 ± 1 ^{ab}	N.D ^{a}
		350	9525 ± 5155 ^{bc}	251 ± 136 ^{ab}	77 ± 8 ^{ab}	N.D ^{a}
		700	5205 ± 3533 ^{abc}	103 ± 69 ^{ab}	358 ± 14 ^{c}	5 ± 0 ^{b}
		1000	8594 ± 3084 ^{abc}	183 ± 74 ^{ab}	5 ± 7 ^{a}	N.D ^{a}
	0.98	0	13,633 ± 2270 ^{c}	542 ± 99 ^{c}	195 ± 135 ^{b}	N.D ^{a}
		350	8076 ± 3053 ^{abc}	313 ± 113 ^{bc}	N.D ^{a}	N.D ^{a}
		700	275 ± 27 ^{a}	11 ± 3 ^{a}	N.D ^{a}	N.D ^{a}
		1000	786 ± 285 ^{ab}	31 ± 12 ^{a}	N.D ^{a}	N.D ^{a}
OV	0.94	0	1614 ± 34 ^{ab}	91 ± 1 ^{ab}	132 ± 8 ^{bc}	3 ± 0 ^{bc}
		350	1496 ± 153 ^{ab}	82 ± 9 ^{a}	98 ± 9 ^{abc}	2 ± 0 ^{abc}
		700	911 ± 72 ^{ab}	49 ± 6 ^{a}	60 ± 2 ^{abc}	N.D ^{a}
		1000	370 ± 59 ^{a}	25 ± 4 ^{a}	N.D ^{a}	N.D ^{a}
	0.96	0	14,136 ± 10,836 ^{abcd}	342 ± 254 ^{abc}	175 ± 5 ^{cd}	2 ± 0 ^{abc}
		350	24,284 ± 3092 ^{d}	664 ± 124 ^{c}	286 ± 71 ^{d}	4 ± 1 ^{c}
		700	20,737 ± 9082 ^{cd}	504 ± 222 ^{c}	278 ± 38 ^{d}	4 ± 1 ^{c}
		1000	15,866 ± 1696 ^{bcd}	339 ± 54 ^{abc}	68 ± 74 ^{abc}	1 ± 2 ^{ab}
	0.98	0	13,671 ± 831 ^{abcd}	551 ± 32 ^{abc}	17 ± 1 ^{ab}	N.D ^{a}
		350	11,751 ± 876 ^{abcd}	472 ± 35 ^{abc}	18 ± 2 ^{ab}	N.D ^{a}
		700	6827 ± 766 ^{abc}	296 ± 36 ^{abc}	N.D ^{a}	N.D ^{a}
		1000	7315 ± 755 ^{abc}	321 ± 22 ^{abc}	5 ± 7 ^{a}	N.D ^{a}

ND: Not detected (values below detection limits).

4. Discussion

Environmental sustainability, as well as ensuring food safety, are important issues which have increased the search for new products that might be applied as fungicides or natural preservatives, to replace synthetic chemicals to control the growth of toxigenic species in agrifood products. It has been widely demonstrated that essential oils (EOs) could be a good alternative to reduce fungal growth and mycotoxin production by several toxigenic species [11]. However, to develop appropriate control strategies to be applied in food matrices, it is important to study fungal behavior under

different environmental conditions. Several authors have reported a variation in the effectiveness of fungicide treatments under different environmental conditions, mainly temperature or humidity [9,25]. In order to establish the interaction of these compounds with environmental factors (i.e., storage or conservation conditions), in vitro ecophysiological studies are a good option since they provide accurate and rapid results. There is also a need for rapid in vitro techniques that give significant information on the range of actions of these compounds under different environmental conditions. The Microbiological Growth Analyzer, Bioscreen-C, is a fast system that allows the evaluation of the effects of these new control agents under a combination of various environmental factors [26]. Moreover, this method has been successfully applied to evaluate the growth of filamentous organisms through automated monitoring [20,27]. In addition, this system provides an inexpensive tool to simultaneously test various compounds and to establish their optimal environmental conditions to be applied. This method allows us to plan large-scale studies since it is composed of two 100-well plates, requiring a minimal volume (300–500 μL/well), and all wells can be treated independently [20]. As mentioned before, the parameter studied in this work was the time to detection (TTD, time in which fungal growth is detected at a certain biomass level), which makes the calculation independent of the experimental time [20]. The study of these parameters is a very good approximation to understand the growth of fungal colonies in a 3D space and at very low biomass levels [10].

Recent consumer trends towards safer foodstuffs, produced using sustainable and ecofriendly methods, have sparked great interest in new alternatives to traditional chemical food preservatives or synthetic fungicides [13]. In a recently published work carried out by our group, the EOs extracted from *Satureja montana* (SM) and *Origanum virens* (OV) were demonstrated to be effective to controlling *A. flavus* growth and its ability to produce aflatoxins (AF) in vitro or in maize grains when humidity was maintained at high levels [28]. Considering the influence of environmental factors on the effectiveness of EOs, the objective of the present work was to determine if they were able to control fungal growth and AF production at three different water activity (a_w) levels. Moreover, it is known that the additional stress posed by fungicide agents may stimulate mycotoxin production as a defense reaction when environmental conditions vary [29]. Our results, which showed that under specific temperature x a_w combinations, the amount of toxin was increased, confirming the necessity of performing this kind of integrated experiment to test the effectiveness of antifungal compounds at different doses and in a wide range of conditions. This is especially important in the case of low-producing strains, such as the one we used (A7), which without EOs would not be considered as a problem in terms of food safety. However, AF production spikes were detected at certain conditions of a_w under the presence of both EOs, increasing the potential risk for consumers.

In this study, we have demonstrated that SM EO was able to retard fungal growth and reduce AF production, mainly at the highest a_w levels tested. The results obtained regarding the effect of SM EO on fungal growth and AF production by *A. flavus* at 0.98 a_w using Bioscreen C are similar to those reported in previous in vitro studies in our laboratory [28]. The most significant results in this case were observed at 0.96 a_w, which is coincident with the conditions in which both isolates reached the

highest levels of AF production. In this latter case, the levels of inhibition reached significant values in the case of fungal growth and AF production, respectively, even at the lowest dose tested (350 μg/mL). Therefore, the application of this EO might be adequate during storage when the moisture levels of the products are quite high. As mentioned above, the treatment with SM EO was not effective to significantly reduce AF production at 0.94 a_w. However, production levels reached at this a_w are quite low, even at control conditions compared with other conditions, which again reveals the relevance of maintaining good storage conditions to avoid the AF contamination of agrifood products, and it would not be necessary to apply any fungicide treatment. Besides, this treatment would be applied in regions with wet weather conditions where the maintenance of these good storage practices is difficult and expensive.

Taking into account our results, the application of SM EO would be appropriate in food products with high water content and those that are frequently contaminated by AFs such as sorghum, almonds, pistachio and rice. On the contrary, the treatment of dried food matrices might not be necessary due to the inability of *A. flavus* to produce AFs in these extreme conditions.

5. Conclusions

Our results demonstrate that the application of *Satureja montana* essential oils might be a good option to prevent aflatoxin contamination of food products, although its effectiveness widely differed among water activity (a_w) levels. At lower a_w conditions, *A. flavus* growth was significantly delayed and aflatoxin production was consistently reduced compared to other humidity conditions, but no effect was observed after essential oil treatment. This means that if it is applied in dry matrices or when good storage practices are applied, it will be an unnecessary cost for the producers. Meanwhile, application in wetter conditions will be adequate as an effective control method to guarantee low levels of aflatoxins.

Author Contributions: All authors conceived the experimental design. M.G.-D. and E.G.-C helped with laboratory analysis. M.G.-D. and A.M. performed statistical analysis and wrote the original draft. B.P., J.G.-S. and N.M. reviewed and edited the manuscript. All authors read and approved the final version of the document.

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Conflicts of Interest: The authors declare no conflict of interest.

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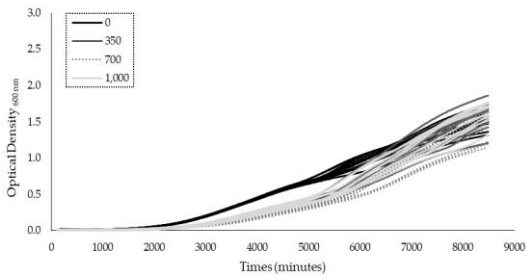
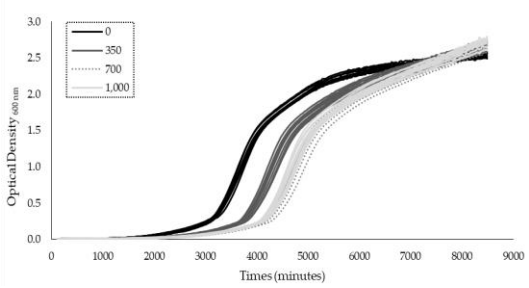
ADDITIONAL INFORMATIÓN

APPENDIX A. Growth curves.

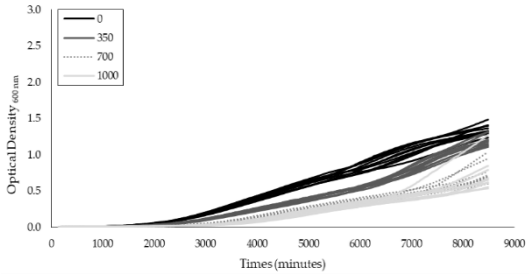
The growth curve obtained in Bioscreen-C for two strains of *A. flavus* (A10 and A7), under the different essential oil concentrations (0, 350, 700 and 1000 µL) of *Satureja montana* (SM) and *Origanum virens* (OV), to the three water activities (0.94, 0.96 and 0.98 a_w) tested.



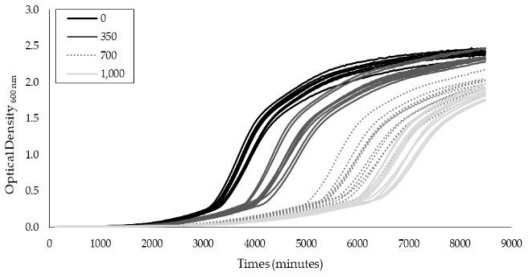
Growth curve A10 strain at 0.94 a_w in OV



Growth curve A7 strain at 0.94 a_w in OV



Growth curve A10 strain at 0.94 a_w in SM



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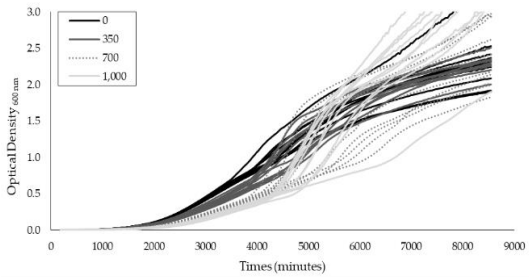
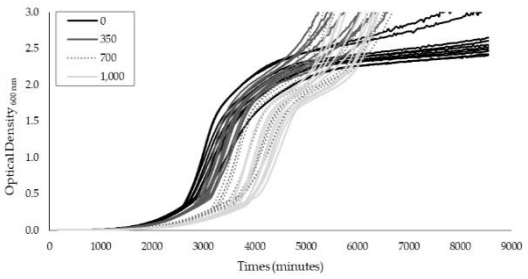


Growth curve A10 strain at 0.96 a_w in OV



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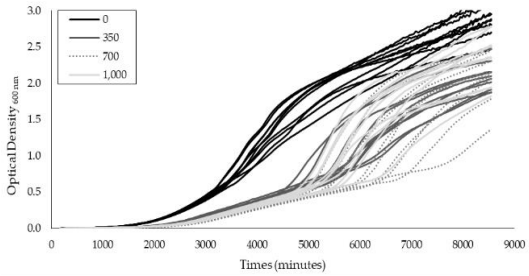
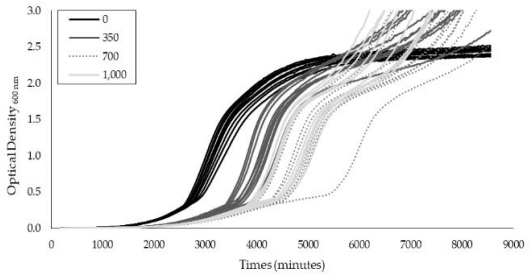
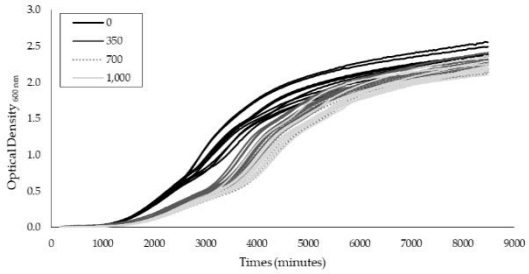


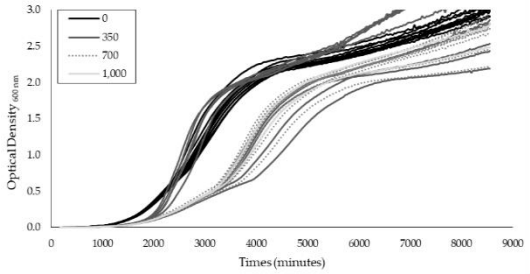
Growth curve A10 strain at 0.96 a_w in SM



Growth curve A7 strain at 0.96 a_w in SM



Growth curve A10 strain at 0.98 a_w in OV



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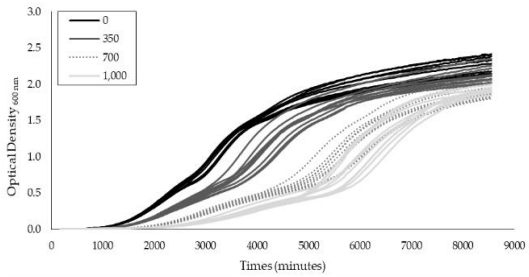
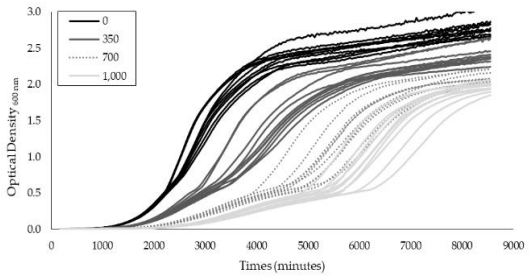


Growth curve A10 strain at 0.98 a_w in SM



Growth curve A7 strain at 0.98 a_w in SM

APPENDIX B. Chromatograms of *Origanum virens* and *Satureja montana* essential oils.

		Area (%)	
Time (minutes)	**Compound**	***Satureja montana***	***Origanum virens***
11,03	tricyclene	ND	1.7
11,04	alpha-thujene	2.0	ND
11,39	alpha-pinene	1.5	0.7
12,01	Canfeno	0.6	0.2
12,63	Sabineno	0.9	0.6
12,98	beta-pineno+Myrcene	5.7	2.4
13,81	delta-3-carene	0.5	0.4
14,18	alpha-terpinene	3.2	4.5
14,49	para cymene	12.6	3.6
14,64	Limoneno	1.6	0.4
15,70	gamma-terpinene	22.5	46.0
16,18	cis sabinene hydrate	0.5	0.3
17,02	linalool	1.6	0.1
20,13	borneol	1.4	0.2
20,32	terpinen-4-ol	1.0	0.5
22,16	nerol	ND	1.3
23,97	thymol	2.0	21.0
24,42	carvacrol	34.6	0.1
28,70	trans-caryophyllene	1.5	5.1
29,84	e-beta-farnesene	0.1	0.9
30,60	valencene	0.2	3.0
31,08	bicyclogermacrene	1.0	2.1

ND: not detected

DISCUSIÓN GENERAL

A nivel mundial los cereales son uno de los principales productos de la dieta, tanto para la alimentación humana como animal, ya que son una fuente importante de energía, minerales y vitaminas [1]. La producción mundial de cereales en la campaña 2018/2019 fue de más de 2.600 millones de toneladas, de las cuales una décima parte (289 millones de toneladas) fue cultivada en la Unión Europea, siendo España el quinto productor comunitario con 23 millones de toneladas**.** La cosecha de cereales en la campaña de comercialización 2018/19 en España fue un 44 % mayor que en la campaña anterior, la cual se había caracterizado por un bajo rendimiento a consecuencia de la sequía. Aun siendo uno de los principales productores comunitarios, la producción nacional no llega a cubrir las necesidades internas del país, por lo que España es un importador neto de cereales, principalmente maíz y trigo blando. La Unión Europea es nuestro principal socio comercial, si bien en el caso del maíz, la mayor parte de las importaciones proceden de terceros países (Ucrania, Brasil y Canadá) [2]. Particularmente, en España existe un déficit para poder elaborar piensos para consumo animal, ya que el maíz es un producto básico para su fabricación por su alto aporte energético [3]

En general, la producción y el número de hectáreas de los diferentes tipos de cereales cultivados en España, se han mantenido estables en las últimas campañas. Sin embargo, la superficie destinada al cultivo de avena ha ido en aumento, así como su producción, que ha pasado a ser en 2018 más del doble de la del año 2012. [2]. Este aumento se debe a una mayor demanda por parte de los consumidores, ya que en las últimas décadas se ha producido un cambio drástico en los hábitos alimentarios. Las enfermedades provocadas por alergias o intolerancias alimentarias, como por ejemplo al gluten, o el estilo de vida actual que ha incrementado la incidencia de patologías como la diabetes y la obesidad, son las principales razones por las que los consumidores optan por dietas más saludables y naturales [4].

Aspergillus y *Fusarium* son dos de los principales géneros fúngicos que contaminan los cultivos de cereal en todo el mundo. A parte de que su presencia en los granos puede disminuir las propiedades nutricionales y organolépticas de los mismos, algunas de las especies que están incluidas en estos géneros pueden producir micotoxinas [5]. En numerosas ocasiones se ha descrito la contaminación simultánea de varias especies toxígenas coexistiendo en los cereales y sus productos derivados [6], lo que puede derivarse en la contaminación de los productos por varias micotoxinas a la vez, pudiendo ocasionar graves problemas para la salud humana y animal [7]

Según un reciente estudio, se estima que alrededor del 25-50 % de los cultivos de todo el mundo están contaminados por algún tipo de micotoxinas [8]. Desde el 2010 al 2018, el mayor riesgo por la contaminación por micotoxinas en Europa se ha detectado en el trigo y en el maíz, ya que se ha visto que presentan altas concentraciones de fumonisinas (FB), deoxinivalenol (DON), aflatoxinas (AF) y zearalenona (ZEA). En algunos casos, incluso se ha demostrado que determinados alimentos derivados del maíz, el contenido de FB_1+FB_2 suele estar por encima de los límites legales [9]. Resultados similares se han observado en la contaminación por

DON, que en muchos casos superaba los niveles máximos establecidos en la normativa para trigo, cebada, maíz y avena. Esta presencia de micotoxinas en los granos de cereal, no solo supone una grave amenaza para la seguridad alimentaria, sino que genera grandes pérdidas económicas a los agricultores y ganaderos [1].

Uno de los cereales más importantes a nivel mundial es el maíz [10]. En España se utiliza principalmente para alimentación animal, bien como forraje o para la fabricación de piensos, y sólo una pequeña proporción se destina a la alimentación humana, tanto para consumo directo como en la fabricación de algunos productos como los copos de maíz para el desayuno, harinas, aceite, alimentos infantiles y el almidón [11]. La avena es utilizada básicamente en la alimentación humana y es consumida en los desayunos de medio mundo en forma de gachas [12]. Durante el siglo XX, la producción de la avena disminuyó a favor de cultivos con mayor rendimiento como el trigo y el maíz, pero como ya hemos comentado, en los últimos diez años se ha incrementado la superficie de cultivo y la producción de avena, debido a su mayor demanda. La Autoridad Europea de Seguridad Alimentaria ha definido las propiedades beneficiosas del consumo de avena como pueden ser la mejora del equilibrio de la glucosa en sangre y una ayuda en la reducción de los niveles de colesterol debido a su alto contenido en fibras solubles y beta-glucanos. Además, la avena es rica en ácidos grasos insaturados, que están relacionados con la reducción del riesgo de enfermedades cardiacas y vasculares. Por otro lado, tiene un alto contenido en polifenoles y avenantramidas con propiedades antioxidantes y antiinflamatorias [13]. Con todo ello, la avena puede contribuir significativamente al mantenimiento de una correcta dieta, y se ha convertido en uno de los cereales preferidos por los consumidores.

Debido a la importancia del maíz y la avena en España, son necesarios estudios como el planteado en esta Tesis para poder conocer la situación actual en España de estos cereales con respecto al riesgo de su contaminación por micotoxinas. Hasta la fecha, hay muchos trabajos sobre la presencia de micotoxinas en el maíz a nivel mundial en el momento de la cosecha o durante el almacenamiento, pero nunca se había estudiado la contaminación por hongos toxígenos y sus micotoxinas a lo largo de las distintas fases del cultivo del maíz. Con respecto a la avena cultivada y comercializada en España, hasta ahora no se había realizado un estudio tan amplio respecto al riesgo de contaminación por micotoxinas.

La contaminación por micotoxinas en los cereales y sus productos derivados es difícil de predecir al depender de muchos factores, pero se sabe que está muy relacionada con las condiciones ambientales durante el cultivo o con operaciones inadecuadas durante los procesos de recolección, almacenamiento o elaboración [14,15]. Por lo tanto, dicha contaminación puede ser minimizada adoptando ciertas medidas preventivas y de control a lo largo de toda la cadena de producción. Estas actuaciones se centran principalmente en el establecimiento de Buenas Prácticas Agrícolas (BPA) e industriales y tienen como objetivo prevenir, eliminar o reducir el riesgo de micotoxinas en los alimentos y piensos, garantizando de este modo la

seguridad alimentaria [16]. Las BPA constituyen la primera línea de defensa para evitar la contaminación de los cereales por micotoxinas, aunque posteriormente es imprescindible el establecimiento de un correcto sistema de Análisis de Peligros y Puntos de Control Crítico (APPCC). Dichos sistemas se han mostrado muy eficaces, pero para su correcta aplicación es imprescindible conocer los factores que fomentan la colonización y el desarrollo de las especies potencialmente toxígenas, y la producción de micotoxinas ya que es el primer paso para conseguir diseñar y aplicar un sistema eficaz, encaminado a reducir al máximo las micotoxinas en los cereales [15]. Por ejemplo durante el almacenamiento del maíz se va a crear un ecosistema artificial, que va a producir cambios en la calidad de los granos debido a interacciones entre factores físicos, químicos y biológicos. Las especies de *Aspergillus* y *Fusarium* pueden contaminar el maíz durante la cosecha y, pueden posteriormente, ver aumentada su producción de micotoxinas si las condiciones de humedad y temperatura durante el almacenamiento no son controladas adecuadamente. Es por ello que para prevenir la aparición de micotoxinas durante el almacenamiento del maíz, es imprescindible el secado de los granos poco después de la cosecha tan rápido como sea posible. Además, hay que evitar daños en los granos antes y después del secado, así como durante el almacenamiento para impedir una fácil colonización por parte de los hongos [17].

En la actualidad no se dispone de técnicas que permitan la eliminación completa de las micotoxinas una vez que están presentes en los alimentos. Por tanto, la Unión Europea, para proteger a los consumidores de este riesgo, ha establecido unos contenidos máximos de micotoxinas en los cereales y sus productos derivados, de modo que, se pueda evitar que cereales muy contaminados ingresen en la cadena alimentaria [18]. Desde el año 2006 que se publicara la primera legislación a nivel Europeo, se han realizado numerosos estudios que han demostrado la presencia de micotoxinas en gran variedad de productos alimentarios, lo que ha supuesto la aplicación de cambios en la normativa para abarcar estos nuevos riesgos.

Con el fin de aportar nuevos datos sobre el riesgo de contaminación por micotoxinas en los cultivos de maíz y avena en España, uno de los objetivos de esta Tesis Doctoral, fue evaluar la presencia de especies del género *Aspergillus* y *Fusarium* potencialmente toxígenas en ambos cereales. Se analizaron muestras de campo de las principales zonas productoras de España recogidas en el momento de la cosecha, así como derivados comerciales adquiridos en diferentes establecimientos españoles. La especie más detectada en campo en los cultivos de avena recolectados por toda España fue *A. flavus* (63 %), mientras que en el maíz se detectaron tanto *A. flavus*, como *F. verticillioides* y *F. proliferatum* en un alto porcentaje (76, 62 y 47 % de muestras contaminadas, respectivamente). Los datos obtenidos en maíz concuerdan con los de otros trabajos realizados en otros países europeos [11,19-21], aunque en el caso de la avena se observaron grandes discrepancias. Distintos estudios han descrito que los granos de avena suelen estar contaminados por especies del género *Fusarium*, principalmente productoras de tricotecenos (TCT), aunque si bien es cierto que la mayor parte de los estudios publicados se han realizado en el Norte de Europa con condiciones climáticas muy diferentes a las de España [22-24]. Se han publicado numerosos trabajos que demuestran que la contaminación de los cereales con

micotoxinas varía según la localización geográfica y las condiciones agroclimáticas de la región donde se cultivan [25]. Las AF, la ocratoxina A (OTA) y las FB se encuentran frecuentemente en la región meridional, que tiene un clima mediterráneo cálido/húmedo, mientras que DON y ZEA van a prevalecer en la región septentrional, con clima más frío [26]. Además, el cambio climático está produciendo un aumento de la temperatura media de los países del Sur de Europa, lo que puede hacer que la presencia de AF aumente significativamente en esta región [27]. Dentro de este contexto, se han publicado distintos modelos que predicen que las AF podrían aparecer en distintos cultivos de cereal en la UE [28]. En esta Tesis, se ha demostrado la presencia de *A. flavus*, potencial productor de AF, no sólo en maíz sino también en avena lo que podría ser un indicador del aumento de la incidencia de este hongo debido al cambio climático.

Por otro lado, el manejo del régimen hídrico de los cultivos es otro factor determinante a la hora de estudiar la distribución de los hongos toxígenos. En general, las exigencias hídricas de *Fusarium* son mayores que las de *Aspergillus* [29], y este puede ser el motivo de que en los cultivos de regadío, como el maíz, las especies de *Fusarium* se desarrollen más fácilmente que en la avena, que en España se cultiva en régimen de secano.

Otro punto a tener en cuenta es que hay pocos trabajos dirigidos a estudiar la presencia de los hongos toxígenos, ya que la mayor parte de ellos se centran en demostrar la presencia de micotoxinas en el producto final. La mayoría de estos estudios señalan que a pesar de que los hongos pueden estar presentes tanto en el campo, como en post-cosecha, la producción de micotoxinas ocurre principalmente durante el almacenamiento cuando las condiciones de secado y almacenamiento no son las adecuadas [30-33]. Es por ello que en esta Tesis se planteó realizar un estudio exhaustivo en el que se analizara tanto la presencia de hongos toxígenos como de sus micotoxinas durante todo el ciclo de cultivo del maíz (floración, pre-cosecha y/o post-cosecha), y determinar en qué momento aparecen estas especies y cuándo empiezan a producir toxinas. Para ello se realizó un estudio durante tres campañas consecutivas (2016-2018) en una explotación de maíz de la provincia del Sur de Madrid, que aplicaba BPA tanto en el campo como en el almacenamiento. La presencia de *A. flavus* fue muy notoria durante todo el ciclo del cultivo desde la floración hasta el almacenamiento durante las tres campañas, pero también se detectaron importantes especies potencialmente toxígenas como *F. verticillioides*, *F. proliferatum*, *F. graminearum*, *A. parasiticus*, *A. carbonarius*, *A. niger* y *A. welwitschiae* en alguna de las etapas. Sin embargo, las únicas toxinas detectadas fueron las fumonisinas B_1 y B_2 (FB_1 y B_2) en muestras de grano antes de la cosecha, y en ningún caso, el sumatorio de las FB alcanzó los límites establecidos por la legislación. Estos resultados confirman lo descrito en otros estudios que destacan la importancia de las BPA en campo, así como el control de las condiciones de almacenamiento para minimizar la contaminación por micotoxinas de los cereales [32-34]. Estos buenos resultados se pueden deber a que en la explotación donde se llevó a cabo el estudio, se han implantado numerosas acciones que se consideran esenciales en la gestión del cultivo como el secado de los granos tras la cosecha, la prevención de daños en los granos durante la cosecha y su manipulación, la correcta elección del momento de la cosecha, así como la disposición de unas buenas

instalaciones de almacenamiento con sistemas de control de humedad y temperatura [35].

Los estudios llevados a cabo durante esta Tesis tienen, por tanto, un gran interés para los agricultores. Conocer qué es lo que ocurre durante el ciclo del cultivo del maíz, cuáles son las principales especies potencialmente toxígenas que contaminan dicho cultivo, así como qué toxinas pueden contaminar el grano, son puntos fundamentales para controlar y reducir el riesgo de micotoxinas en los cereales. La identificación de estas especies en campo es fundamental para proporcionar información sobre qué micotoxinas podrían estar presentes en las matrices alimentarias y así poder aplicar medidas correctivas [36,37]. El análisis de las muestras mediante protocolos de PCR específicos para cada especie realizados en este, y otros estudios llevados a cabo por nuestro equipo, han demostrado ser unas buenas herramientas para predecir el riesgo de contaminación por micotoxinas en los granos de cereal y ofrecen una alternativa rápida, sensible y específica a los procedimientos microbiológicos convencionales en el diagnóstico de hongos.

Otro importante estudio de esta Tesis Doctoral se centró en comprobar la incidencia de hongos toxígenos en muestras derivadas de maíz y avena disponibles en los mercados Españoles y, por tanto, predecir su posible contaminación por micotoxinas, si no son conservados correctamente. En este trabajo, se han detectado distintas especies toxígenas en una gran variedad de productos como palomitas de maíz, kikos o harina tanto de maíz como de avena, lo que podría estar relacionado con una posible contaminación por micotoxinas. Diversos autores han informado del contenido de micotoxinas en productos elaborados a base de cereales aptos para el consumo humano, disponibles en diferentes mercados de países de la Unión Europea [38-40]. El Sistema de Alerta Rápida de Alimentos y Piensos (RASFF, por sus siglas en inglés) desde el 2015 al 2019 ha notificado 40 alertas alimentarias que se correspondían a productos a base de maíz y de avena, tales como palomitas de maíz, harinas, pastas, galletas y cereales para el desayuno, contaminados por niveles que excedían la legislación de AFB_1, FB, DON, OTA, ZEA o toxinas T-2 y HT-2. Además, se declararon 3 rechazos en frontera debido a palomitas de maíz contaminadas con DON y AFB_1 [41]. Estas notificaciones del RASFF relacionadas con productos disponibles en los mercados Europeos, ponen de manifiesto la importancia de realizar estudios como el presentado en esta Tesis en muestras comerciales para poder detectar los productos más susceptibles y garantizar la seguridad alimentaria y la salud de los consumidores.

Como se ha comentado anteriormente, el control de las especies potencialmente toxígenas en las plantaciones de cereal, tanto durante el cultivo, como en el almacenamiento de los granos, es crucial para reducir al máximo la presencia de micotoxinas en el producto final. Tradicionalmente, para controlar y/o reducir el desarrollo fúngico, así como la producción de micotoxinas se han aplicado fungicidas convencionales, estimándose que se utilizan más de 23 millones de Kg/año de estos fungicidas en el mundo [42].

Sin embargo, actualmente su uso se tiende a reducir debido a que producen efectos adversos sobre la salud y el medio ambiente y, actualmente, los consumidores

demandan productos alimentarios seguros y producidos con métodos sostenibles que respeten el medio ambiente [43-45]. El uso de fungicidas naturales como los aceites esenciales (AE) se considera actualmente una estrategia prometedora para reducir la contaminación por micotoxinas. Por tanto, otro objetivo de esta Tesis Doctoral fue optimizar un método de control para reducir el crecimiento fúngico y la síntesis de AF en *A. flavus*, basado en extractos de plantas aromáticas que pudiera ser aplicado durante el almacenamiento de los granos de maíz.

Las propiedades antimicrobianas de los AE frente a diversos microorganismos de interés alimentario, así como sus propiedades antioxidantes han sido estudiadas ampliamente en estas últimas dos décadas, tanto en condiciones de laboratorio como sobre productos alimentarios, como frutas, verduras, cereales, carnes, pescados, productos lácteos o de panadería [46-53]. Distintos trabajos, además, los han descrito como potentes compuestos antifúngicos y se ha visto que son capaces de interferir en la síntesis de micotoxinas [54-56]. Por todo ello, en los últimos años, los extractos de plantas han empezado a considerarse como una opción segura, ecológica, renovable y fácilmente biodegradable para controlar diversos microorganismos en las matrices alimentarias [45]. Además, los AE tienen otras ventajas sobre los fungicidas convencionales como la reducción de las restricciones previas a la cosecha, la posibilidad de su utilización en zonas ambientales sensibles y ser idóneos para todo tipo de agricultura, incluidos los sistemas de producción ecológica [42]. Estos últimos 5 años se han publicado diversos trabajos sobre la actividad antifúngica de distintos AE frente a especies toxígenas de *Fusarium* y *Aspergillus*, que han demostrado que el efecto que presentan no está extendido a todos los AE sino que depende mucho del tipo de planta de la que se hayan extraído, la concentración del extracto, el modo de aplicación o las condiciones ambientales [29,57-60] Todos estos factores han de ser tenidos en cuenta a la hora de optimizar un método de control basado en extractos de plantas.

Los resultados *in vitro* obtenidos en esta Tesis Doctoral demostraron que tanto los AE como algunos de los hidrolatos (HL) extraídos de *Rosmarinus officinalis*, *Thymus vulgaris*, *Satureja montana* (SM), *Origanum virens* (OV), *O. majoricum*, y *O. vulgare* son efectivos reduciendo el crecimiento de *A. flavus* a las máximas concentraciones ensayadas, aunque la eficacia de los AE es mucho mayor que la de sus correspondientes HL. Los principios activos de estos extractos son compuestos químicos, como hidrocarburos naturales (terpenos, monoterpenos y sesquiterpenos) y compuestos oxigenados (alcoholes, ésteres, éteres, aldehídos, cetonas, lactonas, fenoles y éteres de fenol) que son los responsables de las propiedades antimicrobianas [61,62]. Se ha visto que la actividad antimicrobiana de estos extractos está determinada por la proporción de sus componentes y no solo por la cantidad [63,64], lo que podría explicar la mayor eficacia antifúngica observada en el caso de los AE en comparación con los HL [65].

De todos los extractos naturales utilizados en esta Tesis Doctoral, los AE obtenidos de SM y OV fueron los más efectivos, tanto en la reducción de la tasa de crecimiento en *A. flavus* como en su capacidad para sintetizar de AF. Estos AE son ricos en compuestos terpeno-fenólicos y su actividad antifúngica podría deberse a las propiedades de estos compuestos, que debido a su naturaleza altamente lipofílica y a

su bajo peso molecular, son capaces de perturbar la membrana celular del hongo, causando su muerte o inhibiendo la esporulación y la germinación [64,66]. En un estudio realizado recientemente en nuestro grupo, el AE de OV utilizado en esta Tesis Doctoral fue ensayado para evaluar si afectaba a la viabilidad de *A. steynii* y si se producían cambios en la ultraestructura del hongo [67]. Estos ensayos revelaron que dicho compuesto no solo afectaba a la viabilidad celular, sino que modificaba la integridad de la pared, produciendo deformaciones en los tabiques de las hifas del hongo, por lo que serían interesante confirmar si este efecto es similar en *A. flavus*. Además de la inhibición del crecimiento del micelio, se ha observado que la aplicación de los AE de SM y OV produce una reducción de la concentración de AF. Otros trabajos del grupo de investigación, han visto que la aplicación del AE de OV en cultivos de *A. flavus* produce una disminución en los niveles de expresión de genes implicados en la síntesis de AF [68]. Moon y colaboradores (2018) también demostraron que los terpenos interfieren en la ruta de biosíntesis de la AFB_1, disminuyendo la expresión de los genes *aflD* y *aflQ* [60].

Una vez que se seleccionaron los AE de SM y OV debido a su eficacia *in vitro* para reducir el crecimiento de *A. flavus* y la producción de AF, se quiso conocer el efecto de dichos compuestos bajo diferentes condiciones ambientales, para comprobar si podrían ser aplicados como fungicidas sobre las granos de cereal. Varios autores han demostrado que la eficacia de los tratamientos fungicidas puede variar bajo diferentes condiciones ambientales, principalmente cuando se modifican la humedad y la temperatura [69,70]. Teniendo en cuenta la posible influencia de estas condiciones ambientales en la eficacia de los AE, se determinó si los AE de SM y OV eran capaces de controlar el crecimiento de *A. flavus* y la producción de AF también a niveles bajos de a_w. Este estudio demostró que ambos AE fueron capaces de retardar el crecimiento a las tres a_w probadas (0,94, 0,96 y 0,98), siendo mayor el efecto a altos niveles de a_w. El mismo efecto a altas a_w fue observado por Guynot y colaboradores (2005) cuando estudiaron la capacidad fungicida de diferentes AE sobre el crecimiento de hongos de interés alimentario, bajo diferentes niveles de a_w y pH [53]. También se observó un leve aumento en la producción de AF en algunas combinaciones de dosis x a_w, aunque en ningún caso hubo diferencias estadísticamente significativas. Este fenómeno de respuesta a la dosis (hormosis), ya se había descrito anteriormente, cuando se demostró que dosis sub-letales de AE pueden reducir el crecimiento fúngico, y a la vez inducir una mayor producción de micotoxinas como mecanismo de defensa a las condiciones de estrés [66,71]. Los resultados de este ensayo permitieron concluir que la aplicación de estos compuestos podría ser efectiva durante el almacenamiento, cuando los niveles de humedad de los granos de cereal almacenados fuesen elevados, y no se puedan reducir por falta de medios o instalaciones adecuadas.

El último paso en la optimización del uso de AE para controlar la presencia de AF en maíz se centro en el desarrollo de un método adecuado para su aplicación a gran escala. Los AE están formados por compuestos altamente volátiles, por lo que la forma de aplicación sobre los granos de cereal es esencial para maximizar su efecto en el tiempo. Se valoraron diferentes técnicas de encapsulación de AE, ya que diversos autores consideran que son una buena opción para reducir la pérdida de los principios activos, mejorar su biodisponibilidad y aumentar su protección contra los factores

ambientales, especialmente la temperatura, la luz y la disponibilidad de oxígeno [72-74]. De todas las técnicas de encapsulación evaluadas en esta Tesis Doctoral, la novedosa técnica de encapsulación en niosomas fue la más efectiva, a la hora de maximizar el efecto del AE sobre el crecimiento fúngico y la producción de AF en los granos de maíz inoculados con *A. flavus*. Los niosomas, aparte de proporcionar una liberación más controlada de los compuestos activos encapsulados, van a dispersarse en una fase acuosa, de modo que su aplicación puede realizarse fácilmente mediante sistemas de aspersión. De esta manera, se proporciona a los agricultores un eficaz método de control durante los largos periodos de almacenamiento de los granos y que, además, puede ser fácilmente dispersado en los almacenes. Para comprobar el efecto a gran escala, los AE de SM y OV fueron encapsulados en niosomas y aplicados en granos de maíz almacenados en bolsas de poliuretano e inoculados con *A. flavus*, simulando condiciones reales de almacenaje, en las que la humedad y la temperatura no pueden ser controladas. Los niosomas evitaron la perdida de los principios activos de los AE, protegiéndoles de los factores ambientales y asegurando una liberación controlada de estos compuestos antimicrobianos durante el almacenamiento de los granos, controlando tanto el crecimiento fúngico, como la producción de AF hasta 75 días en ambientes con altos contenidos de humedad.

Gracias a los datos obtenidos a lo largo del desarrollo de esta Tesis doctoral, podemos postular una estrategia de control integrado, basada por un lado en la aplicación de las BPA para controlar el crecimiento y la síntesis de micotoxinas en los granos de cereales, y por otra la aplicación de dos extractos naturales (AE de SM o de OV) encapsulados en niosomas, en aquellas regiones de clima húmedo, en las que el mantenimiento de las BPA son difíciles y costosas durante el almacenamiento de los granos de cereal.

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CONCLUSIONES

De los resultados obtenidos en la presente Tesis Doctoral se pueden extraer las siguientes conclusiones:

1. Se han desarrollado con éxito protocolos de PCR específicos de especie para la detección de *Fusarium graminearum*, *F. langsethiae*, *F. fujikuroi*, *F. poae*, *F. sporotrichioides* y *F. temperatum*. Estos protocolos, junto con otros previamente descritos, han permitido detectar las principales especies productoras de micotoxinas de los géneros *Fusarium* y *Aspergillus* directamente en muestras de campo y comerciales de maíz y avena. De esta manera se pone de manifiesto la utilidad de este tipo de herramientas para la detección temprana de especies potencialmente productoras de micotoxinas, lo que es imprescindible para poder aplicar, en caso necesario, los mecanismos de control adecuados.

2. La contaminación por especies potencialmente toxígenas en el ciclo del cultivo del maíz ocurre desde las primeros estadios, destacando la presencia de *A. flavus* en todas las etapas del ciclo desde la floración hasta el almacenamiento de los granos. Otras especies relevantes fueron *A. niger*, *A. welwitschiae*, *F. proliferatum* y *F. verticillioides*. En la finca donde se realizaron los ensayos se llevan a cabo buenas prácticas agrícolas, lo que puede explicar que solo se detectaran fumonisinas y a niveles por debajo de la legislación. Por tanto, esto pone de manifiesto que la contaminación por micotoxinas en los granos de cereal puede ser controlada eficazmente utilizando estas medidas de prevención, aun estando presentes diferentes especies toxígenas a lo largo del ciclo de cultivo.

3. Las especies toxígenas más relevantes detectadas en los granos de maíz y avena cultivados y consumidos en España fueron *A. flavus*, *A. niger*, *F. verticillioides* y *F. proliferatum*, lo que predice una posible contaminación por aflatoxinas, ocratoxina A y fumonisinas tipo B en dichos alimentos, siendo este riesgo menor en avena. Los resultados indican que el riesgo de contaminación por otras toxinas de *Fusarium*, como DON, NIV y las toxinas T-2 y HT-2, es bajo, por lo que no sería necesario modificar la legislación vigente en estos cereales.

4. Los aceites esenciales y los hidrolatos extraídos de las plantas aromáticas *Satureja montana* y *Origanum virens* fueron los más eficaces de los ensayados para reducir tanto el crecimiento de *A. flavus* como la producción de aflatoxinas en un amplio rango de concentraciones. Además, dichos aceites son más efectivos cuando la actividad de agua es alta, por lo que podrían ser aplicados sobre matrices con un alto porcentaje de humedad para controlar la contaminación por aflatoxinas.

5. El mejor método de aplicación de aceites esenciales para el control de hongos toxígenos sobre granos de maíz es la encapsulación en niosomas. Los aceites esenciales de *S. montana* y *O. virens* encapsulados en niosomas son un excelente método de control sostenible para reducir tanto el crecimiento de *A. flavus* como la concentración de aflatoxinas en los granos de maíz almacenados durante largos periodos de tiempo.

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